Regulatory T cells:
molecular requirements for their selection and therapeutic use in autoimmune disease

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Declaration

I declare that this thesis has been submitted by myself, describes my own work and has not been submitted in any other application for a higher degree.

Katy H. Malpass
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Abstract

Regulatory T cells (Tregs), expressing the transcription factor Foxp3, form a key component of peripheral immune tolerance, guarding against auto-aggressive immune responses. Multiple Sclerosis is an inflammatory and demyelinating disease of the central nervous system (CNS) which is largely believed to be mediated by immune components reacting to the self myelin antigens that insulate the nerve fibres. Recent investigations have reported that regulatory T cells are dysfunctional in MS patients; therefore enhancing the regulatory T cell responses in MS is an attractive therapeutic target.

Using the mouse model of MS, experimental autoimmune encephalomyelitis (EAE) we have attempted to develop disease-relevant Treg-based therapies to prevent disease induction. This required an understanding of the antigenic-reactivity of Tregs during disease. Results described in this thesis show that a proportion of Tregs in the draining lymph nodes and CNS were reactive to the disease initiating antigen(s) and could suppress in vitro responses of naïve T cells bearing transgenic T cell receptors, recognising the same antigen. Adoptive transfer of antigen-reactive Tregs suppressed disease induced with the same antigen, but also reduced disease induced with a distinct myelin antigen. Peptide-based tolerance using a high affinity MHC binding peptide analogue expanded and maintained antigen-reactive T cells which were tolerant to antigenic re-stimulation, although these cells did not express Foxp3. Peptide-treated mice showed reduced incidence of disease relapses during EAE induced against a distinct myelin antigen. Thus, while EAE and MS will involve a polyclonal effector T cell response to many antigens, therapeutic targeting of Tregs reactive against one CNS component may be sufficient to reduce disease.

Endogenous expression of myelin autoantigen did not grossly alter the response of antigen-reactive Tregs in the periphery. However, expression of endogenously derived viral superantigen enhanced the proportion of superantigen-reactive Foxp3+ Tregs in the periphery. This observation was extended using exogenous superantigen, suggesting that prolonged exposure to low dose (super)antigen tips the balance of the immune system in favour of regulation. This has implications for the ability to successfully fight infection, as well as for the limitation of auto-aggressive responses and may contribute to the understanding of the hygiene hypothesis.

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Publication

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Abbreviations

Treg	regulatory T cell
Foxp3	Forkhead box P3
CNS	central nervous sytem
MS	multiple sclerosis
EAE	experimental autoimmune encephalomyelitis
TCR	T cell receptor
MHC	major histocompatibility complex
DNA	deoxyribonucleic acid
DP	double positive
SP	single positive
cTEC	cortical thymic epithelium
mTEC	medullary thymic epithelium
RAG	recombinase activating gene
TSA	tissue specific antigen
AIRE	autoimmune regulator
APECED	autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
DC	dendritic cell
APC	antigen presenting cell
TLR	toll-like receptor
CDR	complementarity determining region
SAg	superantigen
SEB	*Staphyloccocus* entereotoxin B
MMTV	mouse mammary tumour virus
Th	T helper
TNF	tumour necrosis factor
GITR	glucocorticoid-induced TNF related-receptor gene
CTLA-4	cytotoxic T lymphocyte antigen-4
mAb	molecular antibody

IBD	inflammatory bowel disease
IDO	idoleamine 2,3-dioxygenase
HEV	high endothelial venule
IPEX	immune polyendocrinopathy and enteropathy X-linked disease
SCID	severe combined immunodeficiency
NOD	non-obese diabetic
GFP	green fluorescent protein
HA	haemagglutinin
OVA	ovalbumin
LAT	linker for activated T cells
Jak	Janus kinase
Stat	signal transducer and activator of transcription
FKH	forkhead
NFAT	nuclear factor of activated T cells
ChIP	chromatin immunopreciptation
NK	natural killer
CFA	complete Freunds adjuvant
HLA	human leukocyte antigen
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
PLP	proteo-lipid protein
BBB	blood-brain barrier
MMP	matrix metallo-proteinase
LT	lyphotoxin
AEP	asparagine endopeptidase
c.p.m	counts per minute
PTx	Pertussis toxin

1 Introduction

Immune regulation is essential to prevent excessive and unwanted immune responses, including immune responses direct against self. Naturally arising Foxp3+ regulatory T cells (Tregs) play a major role in peripheral immune tolerance, demonstrated by the fatal lymphoproliferative and autoimmune manifestation of both humans and mice that lack these cells. Multiple Sclerosis is a demyelinating disease of the central nervous system (CNS) (Hafler et al., 2005), thought to involve immune responses directed against antigens of the myelin sheath that functions to insulate nerves and allow efficient signal transduction. Tregs have been shown to have defective in vitro regulatory functions in patients with MS (Haas et al., 2005; Venken et al., 2008b; Viglietta et al., 2004) and studies from mouse models of the disease have emphasised the importance of Foxp3+ cells in both protection from disease and recovery from episodes of paralysis (McGeachy et al., 2005; Stephens et al., 2005). The aim of this study was to further characterise the regulatory T cells that arise during disease by using mouse models of MS, experimental autoimmune encephalomyelitis (EAE) and to attempt to harness Foxp3+ Tregs therapeutically for the prevention of paralysis. The data from these studies led us to investigate the effect of superantigenic stimulation on peripheral Foxp3+ populations and how encounter with superantigen may affect the balance of the immune system, including its implications for autoimmunity.

1.1 CD4 T cell Biology

To mount a protective immune response the immune system must be able to detect and respond to a vast number of pathogens. Cells of the innate immune system detect general pathogen associated patterns via receptors on their surface to initiate the inflamatory response. The adaptive immune system also use receptors, however these are designed to recognise specific epitopes of the invading organism. T cells represent one arm of the adaptive immune response and express a cell-surface receptor termed the T cell receptor (TCR). The T cell receptor repertoire is estimated to be in the region of 108 (Mason, 1998) while the potential antigenic peptides which the immune system may encounter is proposed to be in the region of 10^{12}-10^{15} (Mason, 1998; Sospedra and Martin, 2006). Thus, the adaptive immune system relies on the receptors of T lymphocytes to become diverse during development and to show a high level of cross-reactivity in order to recognise all the peptides we may encounter in our lifetime (Mason, 1998). T cells will express either CD4 or CD8, co-receptrs required for productive TCR signalling. The expression of the co-receptor determines the fate and function of the T cell (Germain, 2002) and determines the type of major histocompatibility (MHC) molecule the cell may interact with; CD4+ cells are MHC class II restricted, while CD8+ cells are MHC class I restricted (Mazza and Malissen, 2007). CD4+ T cells represent a population of cells that have unique properties in terms of activation, effector function and the ability to 'help' other cells of the immune system, making these cells key players in our immune responses. The TCR expressed by each individual T cell affects the fate of that cell and ultimately affects the immune response each cell can mount (Aliahmad and Kaye, 2006). Selection of T cells bearing a suitable TCR therefore requires a tightly regulated process to remove those cells that recognise self-antigens with high enough affinities to potentially cause auto-aggressive responses, while selecting for a broad diversity of functional T cells which can recognise foreign antigens.

1.1.1 Thymic generation of T cells and the TCR

Progenitor T cells migrate to the thymus from the bone marrow as double-negative (DN) cells i.e. lacking expression of either CD4 or CD8, the molecules which will determine the cell status and function (Germain, 2002). Generation of a functional T cell receptor (TCR) is essential for T cell survival and progression through the thymic selection process (Falk et al., 2001). Expression of the TCRβ chain is the first process to occur, driven by the activation of recombinase activating genes 1 and 2 (RAG1 and RAG2). These initiate the opening and re-joining of DNA to combine the gene segments of the Vβ chain; variable (V), diversity (D) and joining (J) segments (Mombaerts, 1995; Swanson, 2004). The J segment is associated with one of two constant (C) regions, completing the full TCRβ chain (Spicuglia et al., 2006). If the Vβ chain is successfully formed the arrangement of the second Vβ allele is stopped, in a process termed allelic exclusion (Aifantis et al., 1997; von Boehmer and Fehling, 1997). This process is entirely random, such that each T cell generates a Vβ independently to the next T cell, thus forming one step in generating sufficient receptor diversity (Wagner, 2007).

The Vβ chain then associates with the pre-TCRα chain, forming the pre-TCR which is expressed at the cell surface. Expression of the pre-TCR is required for progression through the stages of T cell selection, suggesting a signalling event occurs from successful pre-TCR arrangements (Huang et al., 2005). Indeed, RAG1 and RAG2 become silenced at this point, while the T cell progresses from the double negative (DN) stage to double positive (DP) i.e. CD4+CD8+. The RAG genes are then re-activated to induce TCRα gene rearrangements. TCRα expression differs to TCRβ in that one cell can express more than one TCRα chain (Lacorazza and Nikolich-Zugich, 2004), the implications of which will be discussed later. The pairing of TCRα and β chains completes the TCR and provides antigen specificity to the T cells, required for the selection processes based on antigen recognition.

1.1.2 Repertoire Generation and Central Tolerance

Because TCR rearrangements are random, there exists the possibility that T cells which cannot recognise self MHC molecules or which may recognise self antigens or innocuous antigens (e.g. commensal bacteria) will be generated. The immune system therefore attempts to delete these cells before they exit the thymus in a process termed central tolerance. Central tolerance occurs via two main processes; Positive and Negative Selection (Hogquist et al., 2005). Positive selection ensures that only T cells which recognise self peptide-MHC complexes, expressed on cortical thymic epithelial cells (cTECs), receive survival signals and progress through the selection process. In this way T cell are classed as 'MHC-restricted' in that they will only respond to their corresponding self-MHC; CD4+ T cells recognising peptides presented by MHC class II and CD8+ recognise peptides in the context of MHC class I (Konig, 2002).

Negative selection purges the repertoire of T cells expressing TCRs which recognise self-peptide-MHC (p:MHC) complexes with too high an affinity (Palmer, 2003; Siggs et al., 2006; Wagner, 2007). These cells are induced to undergo apoptosis, up-regulating pro-apoptotic factors such as Nur77 and Bim (Bouillet et al., 2002; Sohn et al., 2007). T cells which fail to detect p:MHC, or respond at too low an affinity to be of use die by neglect. The window of affinity in which T cells are allowed to survive is therefore relatively narrow and only cells expressing TCRs with intermediate affinities are allowed to progress through the thymus. Cells which have responded too strongly to self p:MHC can undergo receptor editing; re-activation of RAG-genes allows the T cell to change the TCRα chain to a less self-reactive TCR (Santori et al., 2002). This process has been suggested to also enhance positive selection, thus increasing the number of T cells in the repertoire. The avidity and affinity with which cells are positively selected has been suggested to determine cell fate decisions, such as wither to become a regulatory vs. effector cell as will be discussed later.

While attempting to rid our bodies of self-reactive cells, the overall process of central tolerance presents a few problems. All T cells have been selected on the basis of recognising self-antigens presented in the context of self-MHC, therefore a degree of self-reactivity must exist in every cell of the immune repertoire. Similarly, the expression of dual TCRs on one cell, by expression of two TCRα chains, also suggests that highly self-reactive T cells may escape deletion if selected on the TCR expressing an α-chain which is not self-reactive or confers a lower affinity for the selecting peptide (Sarukhan et al., 1998). The best available explanation as to why the immune system would risk this problem is that the cost:benefit ratio of these process must be high enough. Firstly, if we were to delete all self-reactive cells in the thymus we would greatly restrict our peripheral immune repertoire (Anderton and Wraith, 2002). Secondly, while expression of dual TCRα chains has been demonstrated to allow the escape of self-reactive T cells from deletion (Zal et al., 1996) it has also been suggested to further extend the TCR repertoire to foreign antigens and contribute significantly to the peripheral TCR diversity (He et al., 2002).

Central tolerance relies on the availability of self-antigens in the thymus that can be presented to developing thymocytes. This is easy to envisage with ubiquitous self-antigens, however the immune system also requires to delete T cells reactive against tissue specific antigens (TSAs) (Kyewski and Derbinski, 2004). These antigens were originally believed only to be expressed at peripheral sites and tolerance to these antigens would be controlled by deletion of the self-reactive T cells in the periphery upon encountering the tissue-specific antigen. However, expression of TSA's has now been identified in the thymus (Heath et al., 1998). Ectopic expression of tissue specific genes was found to largely be confined to medullary thymic epithelial cells (mTECs) (Derbinski et al., 2001; Smith et al., 1997). The expression of these self antigens was latershown to be under the control of a gene termed autoimmune regulator (AIRE). By analysing the mTECs of AIRE-/- vs WT mice it was shown that expression of TSAs is downregulated in mice lacking the gene (Anderson et al., 2002).

Mutations in AIRE are associated with a rare human autoimmune syndrome called autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (Pitkanen and Peterson, 2003) while mice lacking AIRE also develop multi-organ autoimmunity and have a failure in thymic negative selection (Anderson et al., 2002; Liston et al., 2003). Discoveries in AIRE and its functions over the last decade have confirmed that this gene plays an important role in the central tolerance to antigens normally confined to peripheral tissues (Mathis and Benoist, 2007). Although mTECS exclusively express these TSAs, other antigen-presenting cells (APC) in the thymus, namely dendritic cells (DC) have been shown to acquire TSAs from mTECs and can help mediate positive selection (Gallegos and Bevan, 2004). Collectively these processes can delete highly self-reactive T cells while generating a broad repertoire of T cells capable of responding to invading pathogens and mounting a protective immune response upon activation. Central tolerance is not perfect, as evidence of self-reactive cells in healthy individuals has demonstrated. How T cell might escape these processes is discussed later.

1.1.3 T cell activation

Once selected, T cells emigrate to the periphery where they circulate and scan for their cognate antigen. To become fully activated, T cells require a two-step signal process; signal 1 is recognition of the cognate p:MHC complex via the TCR and TCR co-receptors (CD4/CD8 and CD3) while signal 2 is received from co-stimulatory molecules. The cytokine profile released by the APC can also influence T cell activation and is often referred to as signal 3 (Mosmann and Coffman, 1989) These signals are provided by APC, that include macrophages, B cells and DC which take up antigens and present them in the context of MHC.

1.1.4 Antigen Presentation

Antigen can be acquired in a passive process (pinocytosis) or actively via cell surface receptors which bind the antigen and are then endocytosed. Dendritic cells (DC) show the greatest capacity to stimulate T cells in this way, therefore they are classed as professional APC, and they can show many different phenotypes which depend on their location and function (Banchereau et al., 2000). Some DC are located at peripheral sites, e.g. Langerhans cells in the dermis, where they are poised to encounter antigen and become activated in response to danger signals, while other DC are resident in the lymph nodes (Banchereau et al., 2000). Many roles have been described for DC, thus a number of different subsets exist that can carry out unique functions (Shortman and Liu, 2002). These subsets include myeloid DC (mDC), plasmacytoid DC (pDC) and CD8α+ DC, each defined by unique surface marker expression and functions.

Antigens can be directly transported to the lymph nodes via the lymphatics where it will be ingested by lymph node resident APC, or be taken up by APC in the periphery. Uptake of antigen in the steady state (i.e. in the absence of inflammation) does not fully activate APC (Mahnke et al., 2002). T cells that encounter antigen presented by immature DC also do not become activated and in this way DC can promote tolerance to self or innocuous antigens. Under inflammatory conditions recognition of pathogens by innate pattern recognition receptors such as toll-like receptors (TLRs) (Kumagai et al., 2008) drives maturation of DC, involving up-regulation of co-stimulatory and MHC molecules and acquisition of homing receptors for lymphoid organs (Turley et al., 2000). T cells migrating through the body scan the APC and upon encounter with their cognate antigen a crosstalk between the DC and T cell occurs, with the outcome of T cell function determined by the signals it receives from the APC. In this way DC are proposed to be able to tune the T cell response (Pulendran et al., 2001)

Presentation of peptide:MHC complexes to the cognate T cell results in T cell proliferation in the lymph nodes. (Garside et al., 1998). TCR stimulation induces the up-

regulation of CD40L and CD28 that can interact with CD40 and B7.1/B7.2 respectively, which are expressed on the APC. Co-stimulation via CD28-B7.1/B7.2 induces up-regulation of other co-stimulatory molecules, such as inducible co-stimulator (ICOS) on the T cell that can interact with its ligand, ICOSL, on the APC (Lenschow et al., 1996; Sharpe and Freeman, 2002). ICOS stimulation induces further upregulation of CD40L on the T cell and potentiates the activation of both the T cell and APC in a constant feedback loop to amplify the signals (Kroczek et al., 2004). Co-stimulation provides the signals for full T cell activation and IL-2 production which allows autocrine proliferation and activation of other T cells in the vicinity that express the IL-2R (Minami et al., 1993). The absence of co-stimulation, or 'signal 2', results in abortive T cell activation, resulting in un-responsiveness and inability to produce IL-2, a state known as anergy (DeSilva et al., 1991; Jenkins et al., 1991).

1.1.5 MHC Structure and Function

For T cells to recognise antigenic components they must be processed into peptides and presented in the context of MHC, specifically class II MHC for CD4+ T cells. Class II MHC is composed of two chains, the α and β chains, and can bind peptides of up to 20 amino acids due to the open ends of the peptide binding groove (Fairchild, 1998). The genes which encode the MHC molecules are highly polymorphic in that multiple alleles exist for each MHC isoform. One copy of each allele is inherited from each parent, therefore evolutionary diversity increases the chance that at least a few individuals will possess alleles which are capable of binding and presenting peptides from any pathogen encountered. Further diversity involves mutations in the nucleotide sequences which code for the peptide binding domain and therefore affect the ability and orientation of peptide binding. Many diseases and disorders have been linked to MHC alleles, such as HLA-DR molecules in multiple sclerosis (Holmes et al., 2005) and Type-1 Diabetes (Wong and Wen, 2003).

1.1.6 TCR Structure and Function

TCRs are composed of two chains as mentioned previously, the alpha and beta chains. Each chain possesses a constant (C) and variable (V) region. The Vα and Vβ TCR domains are structurally related to the immunoglobulin V domains on B cells and possess peptide loops termed complementarity-determining regions (CDRs) which contain the peptide/MHC binding sites (Bentley and Mariuzza, 1996). There are three CDRs on the V region of each chain. CDR1 and CDR2 are encoded in the V gene segment and largely bind to sequences on the MHC molecules. CDR3 is formed during site-specific Vα-Jα, or Vβ-Dβ-Jβ recombination during thymic selection. All of the CDR regions show diversity in their sequences; however CDR3 shows the highest diversity due to the random mutations which occur at this site. CDR3 regions recognise peptide residues and the greater diversity in this region therefore enhances the potential for TCRs to recognise a wider range of peptides (Mazza and Malissen, 2007). The orientation of the TCR on p:MHC has been proposed as a two step model; CDR1 and 2 bind the α-helices on the MHC molecule initially, which then allows alterations in the CDR3 region to maximally recognise the peptide (Wu et al., 2002).

1.1.7 Superantigen-mediated T cell stimulation

It has been known for a number of years that certain endogenous and exogenous molecules can act as potent inducers of T cell proliferation and these have been termed superantigens. Some exotoxins that are secreted by gram positive bacteria, for example Staphylococcal enterotoxins (SE's), have been described to have superantigen activity and have been extensively studied over the past few decades (For a review see (Herman et al., 1991).

T cell stimulation with superantigens requires MHC expression on APC, but does not show the requirement for antigen processing or MHC restriction as 'classical' antigen

presentation (Figure I). Superantigens function by simultaneously binding with class II MHC and TCR molecules, but the interaction with MHC molecules occurs outside of their peptide binding grooves (Sundberg et al., 2007). Although the MHC allele is not important, superantigen interactions show differential binding to the class II MHC isotypes. Most murine superantigens bind with a higher affinity to I-E than to I-A and there is a similar hierarchy with human class II and superantigen binding (Herman et al., 1991). The interaction of superantigen with the T cells is mediated by the TCR Vβ chain, shown by the clonal elimination of thymocytes bearing superantigen-reactive TCR Vβ chians in mice expressing certain self-superantigen/MHC combinations (Kappler et al., 1987).

Staphylococcus aureus enterotoxin B (SEB) is one such bacterially derived superantigen, and has been shown to stimulate T cells bearing Vβ8+ TCRs (White et al., 1989). SEB administration to neonatal mice leads to the expansion of of Vβ8+ T cells, followed by the deletion of the majority of those cells after 4 days (Kawabe and Ochi, 1991). The remaining cells have been shown to be anergic and potentially regulatory (Germain, 2008). Similarly, SE stimulation in humans has been shown to be Vβ-dependent (Kappler et al., 1989).

Endogenous superantigens also exist. Kappler and colleagues (Kappler et al., 1987) demonstrated that T cells bearing Vβ17a responded to splenocytes from I-E+ mouse strains by a B cell-derived superantigen, presented by I-E molecules. Subsequent analysis of I-E+ mouse strains showed that the periphery was devoid of Vβ17a+ T cells due to their thymic deletion induced by superantigen encounter. Murine endogenous superantigens arise from the mouse mammary tumour viruses (mmtv's); these are encoded within the mouse genome and maternally inherited (Marrack et al., 1991). The expression of certain mmtv's causes the deletion of mmtv-reactive T cells expressing particular TCR Vβ chains, similar to exogenous superantigens. Vβ5+ T cells are deleted in mice expressing mmtv-9 and are I-E+ (Fink et al., 1992). However, the deletion of

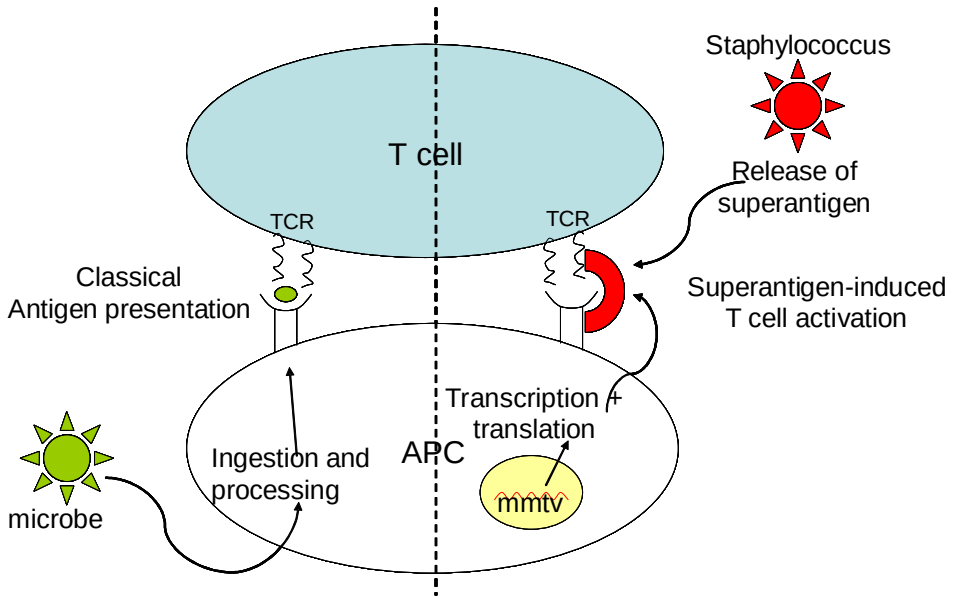


Figure I: Schematic comparison of classical antigen presentation vs. Superantigen presentation to T cells.

Table I: Comparison of the characteristics of Superantigen vs. peptide antigen presentation

	Superantigens	Protein antigens
Antigen Processing	Not required	By APC, internally in vesicles
Binding to MHC class II	Binds outside of antigen groove	Binds in the peptide groove
Interaction with TCR	Site specific interaction with TCR Vβ chain	Site specific interaction with CDR loops of TCR Vα and βchains

Vβ5+ T cells does not occur intrathymically in mice that lack I-E, but they slowly disappear from the periphery (Fink et al., 1994).

Encounter with superantigens, endogenously or exogenously derived, will therefore have a major impact on the peripheral T cell repertoire. The deletion of an entire segment of the CD4+ T cell repertoire has important consequences on the ability to mount an effective immune response. While deletion of superantigen-reactive cells will protect the host from the systemic shock associated with toxic bacterial superantigens it may also determine susceptibility to diseases where a bias in TCR usage has been documented (Acha-Orbea, 1991).

1.1.8 Effector Lineages of CD4+ T cells

CD4+ T cells are named T helper (Th) cells due to their ability to 'help' B cells and CD8+ T cells to become fully activated. In this way, CD4+ cells can be seen as a highly important cell type, central to the adaptive immune response. Th cells were originally classified as either a Th1 or Th2 phenotype (Mosmann and Coffman, 1989). Dendritic cells have the ability to produce many of the cytokines responsible for driving the Th cell lineages. Different signals received by the DC condition the cytokines each cell will make. This is also affected by the phenotype of the DC and therefore these cells can provide the third signal required for T cell activation and polarisation (Kaiko et al., 2008).

CD4+ T cell differentiation towards a Th1, Th2 or Th17 fate occurs under the influence of distinct cytokines and transcription factors (Figure II). Th1 polarisation is driven mainly by IL-12 and the activation of the transcription factor T-bet (Rao and Avni, 2000). Th1 cells produce IL-2, IFNγ and TNF, cytokines that are associated with bacterial infections and macrophage activation, but also in chronic immune responses that can be detrimental to the host (Peluso et al., 2006). Naïve CD4+ cell are driven

towards a Th2 fate by IL-4 and IL-13 and the up-regulation of the transcription factor GATA-3 (Rao and Avni, 2000). Th2 cells produce IL-4, -5 and -13 and are classically associated with parasite infections and B cell activation but are also responsible for the overt immune responses in asthma and allergy (Doherty and Broide, 2007). However, it is becoming clear that there are many overlapping functions of these cell types.

Recently, a new population of helper T cells was discovered that produced IL-17 upon differentiation with IL-6 and TGFβ (Bettelli et al., 2006a). These were termed Th17 cells and are maintained by the cytokine IL-23 which shares a common subunit with IL-12, IL-12p40 (Oppmann et al., 2000). The phenotype is driven by the transcription factors RORγT and STAT3 (Figure II), while it is inhibited by IL-2 (Laurence et al., 2007). Th17 cells produce IL-17A, IL-17F, IL-21, IL-6 and TNFα (Langrish et al., 2005) and have been discovered to be involved in many of the pathologies classically associated with Th1, including autoimmune disease (Cua et al., 2003; Murphy et al., 2003; Ouyang et al., 2008).

1.1.9

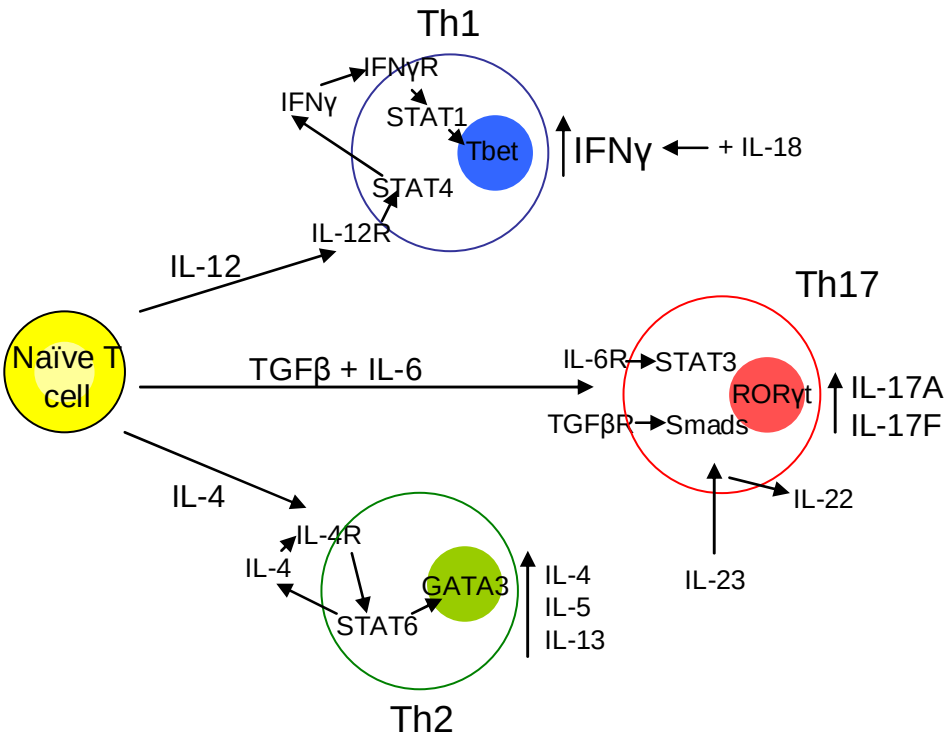


Figure II: Signals required to induce T helper cell differentiation. IL-12 drives the differentiation of Th1 cells, amplified by IFNγ and Tbet transcription. TGFβ and IL-6 drive Th17 cell differentiation via the transcription factor RORγt. IL-4 polarises naïve T cells towards a Th2 cell fate via the induction of GATA3. Differentiation towards one subset represses the induction of opposing transcription factors. Furthermore, cytokine production by one subset blocks signalling via the cytokines released from other subsets, thus enhancing the polarisation towards a distinct Th population.

(Adapted from Stockinger and Veldhoen, Current Opinnion in Immunology 2007).

1.1.9 Peripheral T cell Tolerance

Clearly, while maintaining a diverse immune repertoire allows us to respond to many foreign pathogens, the immune system will generate self reactive T cells. While many of these are purged from the system during thymic negative selection it is now clear that self-reactive T cells exist in the periphery. Every T cell in our bodies has been selected on the basis of self-reactivity. However for most cells this must have been of low enough affinity to escape deletion. Peripheral mechanisms of tolerance must therefore exist to ensure that these cells do not overtly respond to self (Walker and Abbas, 2002).

1.1.9.1 Clonal Ignorance

In some cases, self reactive T cells may exist in the periphery, but never encounter their cognate antigen, and do not become activated. This is termed clonal ignorance (Miller and Heath, 1993). Sequestration of antigen has been shown to occur in organs that have been termed ‘immune privileged’, such as the eye, testis and CNS (Simpson, 2006). Inflammation in these organs would be detrimental to the function of that organ and thus immune responses are prevented by ignorance, although active deletion at these sites has also been documented (Brabb et al., 2000).

Self-reactive T cells may escape from the thymus if the antigen is not presented during the processes of positive and negative selection. Processing of antigen for presentation involves the specific cleavage of proteins at enzymatic sites. This results in the preferential presentation of immuno-dominant epitopes to the T cells (Sercarz, 2002). T cells reactive to epitopes of self antigen that are not presented in the thymus (often referred to as cryptic epitopes) will escape into the periphery. However, as the cryptic antigen is not presented in the periphery, the self-reactive T cells never become activated.

1.1.9.2 Deletion

As in the thymus, if T cells encounter antigen presented by activated DC with too strong an affinity then they are induced to undergo apoptosis, also known as activation-induced cell death (AICD) (Krammer, 2000). Mutations in the gene encoding Fas (CD95) was shown to be responsible for the lympho-proliferative disorder in lpr mice (Watanabe-Fukunaga et al., 1992). Later it was found that the mature T cells in mice with defects in Fas showed a defect in activation induced death and resistance to TCR mediated tolerance (Russell et al., 1993). Furthermore, gld mice that presented with a similar lympho-proliferative phenotype to lpr mice, were shown to have mutations in the ligand for Fas, FasL (Bossu et al., 1993; Takahashi et al., 1994). Mature T cells in the periphery have been shown to up-regulate Fas (CD95) upon strong ligation of their TCR (Ju et al., 1995) and cell death is then be mediated by the binding of Fas to FasL expressed on the same cell or on a neighbouring cell. In other cases, T cell which do not receive the appropriate survival signals will undergo activated cell autonomous death (ACAD) or death by neglect (Hildeman et al., 2002).

1.1.9.3 Anergy

Another outcome of peripheral-self recognition is anergy. Mature T cells will encounter many self antigens in the absence of inflammation, thus will recognise peptide:MHC complexes on DC in the absence of CD28/B7 co-stimulation. This results in abortive T cell activation. Dendritic cells that present antigen in the absence of inflammation are tolerogenic (Shortman and Heath, 2001). Upon secondary exposure to the same antigen proliferation and IL-2 production is blocked and the cells are termed anergic; however anergy can be overcome by the addition of IL-2 (Schwartz, 2003). The induction of anergy in the absence of co-stimulation results in distinct signalling pathways compared to TCR stimulation on the presence of co-stimulation to mediate specific unresponsivess of the T cells (Appleman and Boussiotis, 2003).

1.1.9.4 Active regulation

Over the past few decades evidence has been gathered that distinct, naturally arising cell populations exist to regulate immune responses. These include naturally arising CD4+ Tregs and CD8+ Tregs. However, the induction of regulatory cells in response to antigenic stimulation has also been documented.

The focus of immune regulation has largely been on a population of naturally arising CD4+ regulatory T cells (nTregs) since their discovery by Sakaguchi et al. in 1995 (Sakaguchi et al., 1995) and identification of the transcription factor Foxp3, which is essential for the function of these cells (Fontenot et al., 2003). This population of Tregs and other regulatory cell populations are discussed in the following section, with particular emphasis on Foxp3+ Tregs for the purpose of this thesis

1.2 Regulatory T cell Biology

Certain cells have regulatory properties which can actively suppress immune responses. These include a lineage of T cells which are selected in the thymus to become regulatory cells, or Tregs.

1.2.1 Discovery of natural Tregs

In the early 1970's Gershon and Kondo introduced the idea of 'infectious tolerance', after discovering that thymically-derived T cells may also negatively regulate immune responses in an antigen-specific manner (Gershon and Kondo, 1970; Gershon and Kondo, 1971). This was followed by numerous publications demonstrating suppression of immune responses in vitro by CD4+ and CD8+ T cells, termed suppressor T cells (Tsup) (reviewed in (Germain, 2008)). Identification and function of these cells remained elusive and as techniques developed over the next decade the evidence for Tsup cells was questioned and they rapidly fell out of favour. However, in the early 90's studies into autoimmunity demonstrated that transfer of cells lacking activation markers into immunodeficient (athymic) hosts could cause autoimmunity, while cells expressing low levels of CD45RB (OX-22), or memory-like cells, could prevent disease (Powrie and Mason, 1990). These studies began to suggest that particular subsets of T cells did indeed have the capacity to suppress self-reactive T cells.

It has been known for many years that mice which undergo thymectomy at, or before day 3 after birth succumb to organ-specific autoimmune pathologies (Nishizuka and Sakakura, 1969; Taguchi and Nishizuka, 1987). Similarly, thymectomy at a later stage in development accelerated the onset of diabetes in the non-obese diabetic (NOD) mouse strain (Dardenne et al., 1989). Sakaguchi and colleagues (Sakaguchi et al., 1995) identified that T cells expressing the high affinity IL-2 receptor (IL-2Rα), CD25, and

constituting 5-10% of peripheral CD4+ T cells played a major role in self-tolerance. Thymectomised mice show reduced CD25+ cells in the periphery and transfer of CD25+ cells immediately after thymectomy prevented the autoimmune disease, while CD25- cells did not (Asano et al., 1996). Furthermore, autoreactive T cells could be expanded from CD4+ T cells isolated from healthy, human peripheral blood when the population was depleted of CD25+ cells (Venken et al., 2007). These studies concluded that CD25+ cells which arise in the thymus at day 3 after birth were responsible for peripheral tolerance by maintaining auto-reactive CD25- cells in a dormant state. CD4+CD25+ Tregs were later demonstrated to suppress both CD4+ and CD8+ responses in vitro by inhibiting IL-2 production (Itoh et al., 1999; Thornton and Shevach, 1998), although even now, a decade later, the full mechanism of Treg-mediated suppression is not fully comprehended.

1.2.2 Identifying Treg-specific markers

Initially, CD25 was the best defined marker of Tregs, but as CD25 is expressed by most T cells upon activation attempts to identify other markers of regulatory T cells were therefore warranted.

1.2.2.1 GITR

CD25+ cells isolated from naïve mice were shown to express high levels of glucocorticoid-induced TNF receptor-related gene (GITR) through gene expression analysis (McHugh et al., 2002). It was also demonstrated that stimulation of GITR, via GITR-specific monoclonal antibodies (mAb), along with TCR stimulation could block Treg mediated suppression and induce multi-organ autoimmune disease (Shimizu et al., 2002). GITR knock-out (GITR-/-) mice have no defects in Tregs suggesting that while this molecule may have a role for Treg suppression it is not essential. However, GITR+

cells were shown to prevent colitis induced by CD4+CD45RBhi when co-transferred into SCID mice (Uraushihara et al., 2003). Notably, both CD25+GITR+ and CD25-GITR+ cells demonstrated the same suppressive activity, further emphasising that CD25 is not a definitive Treg marker. More recently multi-organ autoimmune disease could be induced in athymic, nude mice by transfer of T cells depleted of GITRhi cells via anti-GITR mAb (Ono et al., 2006). Overall these data suggest that GITR is expressed by cells which have regulatory potential but perhaps again is not a definitive marker of natural Tregs.

The function of GITR had been suggested to be via modulation of Treg suppression. GITR is largely expressed on activated Tregs (McHugh et al., 2002) perhaps functioning as an 'off switch' for Tregs in situations where inflammation has been resolved or where a robust immune response is required. Indeed anti-GITR mAbs are now being used to promote Treg down-modulation where immune response would be beneficial, for example in tumours and enhancing vaccine responses (Cohen et al., 2006; Ko et al., 2005). Also by blocking GITR-triggering it is possible to prevent Tregs from being turned off in order to maintain immuno-suppression and prevent excess inflammation (Nocentini et al., 2008).

1.2.2.2 CTLA-4

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4 or CD152), has been demonstrated to function as a negative regulator of CD28 signalling and IL-2 production by binding to the B7 molecules on APC (Walunas et al., 1996). Like CD25, it is constitutively expressed on Tregs, but is also up-regulated by naive T cells after activation (Read et al., 2000). The role of CTLA-4 in Treg-mediated suppression was demonstrated using anti-CTLA-4 mAb Fab fragments, preventing its interaction with its ligand (Tang et al., 2004a). CTLA-4 was shown to be important in the control of inflammatory bowel disease (IBD) (Read et al., 2000) and type-1 diabetes (Luhder et al., 1998). Tregs from

CTLA-4-/- mice develop normally and show normal homeostasis and maintain their suppressive ability in vitro. However CTLA4-/- mice still develop fatal autoimmune disease, suggesting this suppression is not effective in vivo (Sakaguchi et al., 2006).

CTLA-4-B7 interactions have been shown to occur between Tregs and APC, but also suggested to occur directly between Treg-Teff. The effect on dendritic cells was shown to be via tryptophan metabolism, specifically the induction of indoleamine 2,3-dioxygenase (IDO) (Fallarino et al., 2003). This enzyme catalyses tryptophan degradation, an essential amino acid for T cells, and ultimately regulates the T cell response by inducing apoptosis (Mellor and Munn, 2004). CTLA-4 interaction with Teff cells has been shown to play a major role in Teff suppression in vitro and in vivo using B7-/- T cells (Paust et al., 2004). Transduction of full length B7, but not a truncated form which lacked the cytoplasmic tail, restored regulation. These results suggested that the CTLA-4-B7 interaction transduced a negative signal to the Teff cells. Blockade of CTLA-4 is therefore a promising target to downregulate Treg function. Indeed, this has already been investigated in clinical trials of patients with metastatic melanoma (Phan et al., 2003). While some of the patients treated with CTLA-4-blocking agents showed regression of the cancer, others showed autoimmune manifestations, demonstrating the delicate balance of regulation in the immune system, something we must consider when attempting to target Tregs in therapy.

1.2.2.3 Other Treg surface markers

CD62L, or L-selectin, is expressed on naïve T lymphocytes and binds to vascular addressins on endothelium such as on the high endothelial venules (HEV) in the lymph nodes. This allows homing of T cells to lymphoid organs. It was recognised early on that T cells expressing CD62L also had suppressive potential (Herbelin et al., 1998) and the CD25+ Tregs which were found in the periphery also expressed CD62L (You et al., 2004). It was demonstrated that specifically CD62Lhi Tregs played a role in tolerance to

autoimmune diabetes (You et al., 2004) and graft acceptance (Taylor et al., 2004). The expression of CD62L by Tregs enhances their homing to secondary lymphoid organs and this may increase the ability of these cells to interfere with T cell activation at this stage. Accordingly, CD62L expression may not enhance Treg function, but rather allow these cells to preferentially locate with effector T cells in the lymph nodes. However, one investigation demonstrated that while CD4+CD25+CD62L^{lo} were also anergic and expressed other Tregs markers, CD62L^{hi} Tregs were more potent suppressors in vitro and maintained their suppressive function better that CD62L^{lo} or un-fractionated CD4+CD25+ cells (Fu et al., 2004). Accordingly, while CD4+CD25+ cells are readily used in many groups as 'purified Tregs' upon sorting from lymphocyte populations, further sorting into a CD62L^{hi} population routinely gives a purer population of Tregs in our hands (Leigh Stephens, manuscript submitted).

CD103 is an αE integrin and is expressed by subsets of CD4+ and CD8+ T cells, but also some DCs, particularly in the intestine (Andrew et al., 1996). CD103 binds to E-cadherin expressed by epithelial cells, particularly in the gut, therefore CD103 is suggested to be a gut-specific homing molecule (Cepek et al., 1994). CD103+ Tregs have been identified in the gut and this subset of Tregs can control wasting disease, particularly by production of the cytokine IL-10 (Banz et al., 2003). Another model demonstrated that only a CD103+ population of Tregs could control a cutaneous model of Leishmania major infection, with the function of CD103 attributed to retaining of Tregs at the infection site (Suffia et al., 2005). However, CD103+ Treg populations have been isolated from both CD25+ and CD25- CD4+ T cells (Lehmann et al., 2002), suggesting these may not always represent a naturally arising Treg cell lineage but an induced Treg cell as will be discussed in section 2.8.

Huehn and colleagues (Huehn et al., 2004) proposed that expression of CD103 and CD62L on Tregs may allow distinction of Tregs at different stages of development. CD25+CD62L^{hi} cells represent naïve Tregs which preferentially home to lymphoid organs, enhanced by expression of the chemokine receptor 7 (CCR7), while CD25+ (and

CD25-) CD103+ Tregs represent effector/memory-like Tregs which preferentially home to peripheral and inflamed tissues by expression of receptors such as CCR2, CCR4 and CXCR3 that attract these Tregs to the site of inflammation. Accordingly, this group demonstrated that the CD103+ Treg subsets preferentially migrated to the inflamed joint in antigen-induced arthritis (Huehn et al., 2004). CD103+ Tregs have also been identified in the inflamed CNS during experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (McGeachy et al., 2005) suggesting that CD103- expression on Tregs may identify a population which is particularly associated with inflammation and may be more potent in this setting.

1.2.3 The Discovery of Foxp3 as a Treg-specific transcription factor

Scurfy mice are a mutant strain where males are grossly affected as the gene harbouring the mutation is X-linked. These mice exhibit increased T cell activation and responses (Clark et al., 1999), lymphadenopathy, splenomegaly and show massive lymphocytic infiltrates in many organs (Godfrey et al., 1991). The characteristics of these mice demonstrate that the scurfy mutation manifests as an autoimmune disease. The disease in scurfy mice is remarkably similar to the human disease X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome (IPEX) (Wildin et al., 2001). In 2001 Brunknow and colleagues (Brunkow et al., 2001) identified the gene which is mutated in the scurfy mouse strain as Foxp3, a new member of the forkhead family of transcriptional regulators. It quickly followed that this same gene was responsible for the human disease (Bennett et al., 2001). These discoveries sparked massive interest in Foxp3 and its function.

In initial experiments, mice that over-expressed Foxp3 showed massively reduced peripheral T cell numbers, and were un-responsive to in vitro stimulation as demonstrated by lack of proliferation or IL-2 production (Khattri et al., 2001; Schubert

et al., 2001). Later it became apparent that the Foxp3-transgenic mice showed reduced T cell-dependent immune responses by somehow regulating the function of effector T cells (Kasprowicz et al., 2003). A major breakthrough came when several labs simultaneously identified that Foxp3 was specifically involved in the control and development of CD4+CD25+ regulatory T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Using mixed bone marrow chimeras of wild-type and Foxp3-/- mice, Fontenot et al. (Fontenot et al., 2005a) found that only cells from the WT mice expressing the Foxp3 gene generated CD4+CD25+ Tregs demonstrating that Foxp3 is indispensable for Treg development.

Furthermore, retroviral transduction of Foxp3 into naïve CD4+CD25- T cells resulted in up-regulation of Treg markers and acquisition of suppressive function (Fontenot et al., 2003; Hori et al., 2003). The in vivo function of Foxp3-transduced cells was shown when these cells were co-transferred with CD4+CD25- cells to severe combined immuno-deficient (SCID) mice. The co-transferred mice were protected from inflammatory bowel disease (IBD) compared to when CD25- cells were transferred alone (Hori et al., 2003). Using antigen-specific, transgenic CD4+ T cells transduced with the Foxp3 gene, Jaekel et al (Jaeckel et al., 2005) demonstrated that these Tregs could induce protection from diabetes in the non-obese diabetic (NOD) model, however this was not achieved using Foxp3-transduced polyclonal CD4+ cells from the NOD mouse, suggesting that the frequency of antigen-specific Tregs may ultimately affect the control of autoimmunty.

The discovery of Foxp3 was a substantial leap in Treg biology as these cells could now be identified as ‘true’ Tregs. However the intracellular expression of Foxp3 presented a problem for identification purposes. The generation of Foxp3-GFP mice by the Rudensky lab overcame this problem (Fontenot et al., 2005b). Using a knock-in allele which possessed an in-frame green fluorescent protein reporter (GFP) fused to the Foxp3 gene, Foxp3+ cells could be detected by confocal microscopy and by flow cytometry without the need for cell lysis. From studies using these mice it was realised

that Foxp3 is expressed exclusively in αβ T cells, 97% of which also express CD4. However, Foxp3 is not exclusively confined to the CD4+CD25+ subset, as approximately 60% of CD4+CD25- T cells in the spleen of naive mice expressing the gene. Both the CD25+ and CD25- Foxp3+ populations demonstrated in vitro suppressive capacity and expressed genes encoding previously identified Treg markers, such as CD103 and CTLA-4 (Fontenot et al., 2005b).

The essential role of Foxp3+ Tregs in preventing autoimmunity was demonstrated in mice which were specifically ablated of Foxp3+ Tregs (Williams and Rudensky, 2007). Thus far, the data on Foxp3 identified this gene as a lineage specification factor for Tregs, similar to the role of T-bet, GATA-3 and RORγ-t in Th1, Th2 and Th17 cells respectively. Further characterisation of Foxp3 expression and how it functions will be discussed in later sections.

1.2.4 Thymic generation of Tregs

The evidence that Tregs represent a functionally and developmentally distinct T cell lineage which arises in the thymus and could regulate immune responses existed even before the identification of Foxp3 (Itoh et al., 1999). Thymic selection was largely viewed in terms of two processes, positive and negative selection (as discussed in section 1.2.), however evidence began to show that the thymus was involved in selection of Tregs. A review by Seddon and Mason in 2000 suggested the generation of Treg cells as the 'third function' of the thymus (Seddon and Mason, 2000).

1.2.4.1 Antigenic-reactivity of Tregs

The processes in the thymus are based on self-reactivity. Negative selection deletes T cells with a high avidity for self-antigen, while positive selection allows survival of

weakly self-reactive T cells. However understanding of the antigenic-reactivity of Tregs and the selection events these cell undergo in the thymus was lacking. The activity of Tregs has been shown to require stimulation via the TCR to exert suppression in vitro and to control organ-specific autoimmune disease in vivo (Thornton and Shevach, 2000; Walker et al., 2003). Also, as antigen-specific Tregs were responsible for the prevention of diabetes in the NOD mouse model (Jaeckel et al., 2005) it was hypothesised that Tregs would express TCRs reactive to self antigens.

Transgenic mice expressing a single TCR and which are crossed onto the RAG-/- background, i.e. cannot undergo TCR revision, rapidly develop spontaneous autoimmunity (Lafaille et al., 1994; Van de Keere and Tonegawa, 1998). RAG-/- TCR transgenic mice were shown to lack CD4+CD25+ cells in the periphery as a result of the deficiency, suggesting that Treg development required receptor editing (Hori et al., 2002) and together these data further suggested a role for TCR specificity in thymic generation of Tregs. Using a TCR transgenic model where all T cells were reactive to an epitope of influenza heamagluttinin (S1 HA) crossed with an HA-expressing mouse strain, Caton's group investigated the role of self-antigen in development of Tregs (Jordan et al., 2001). This study demonstrated that interaction with self-peptide on radio-resistant thymic epithelial cells could select cells to become CD4+CD25+ Tregs. Only T cells which showed a high affinity for the antigen were selected to become regulatory cells as low affinity T cells did not become Tregs. Using a similar model with OVA-expressing D011.10 TCR transgenic mice (all of the TCRs on CD4+ cells are reactive to an OVA peptide), Walker et al. demonstrated increased transgenic Treg numbers in the periphery of OVA-expressing mice compared to non-OVA expressing mice (Walker et al., 2003).

In both of these studies the investigators demonstrated induced Treg generation in the periphery of RAG-/- TCR transgenic mice by introducing the cognate ligand of the TCR into the mouse. These findings explained the deficiency of Tregs in RAG-/- mice which did not express the cognate peptide – these mice lacked a peptide which the Tregs could

be selected upon and therefore were required to undergo TCR re-arrangements to generate a TCR capable of recognising a thymic peptide. RAG-/- mice could not undergo TCR rearrangements, therefore lacked Tregs. Using Foxp3-GFP mice it was confirmed that Foxp3+ thymocytes were restricted to the CD4 single-positive (SP) population and absolutely dependent on TCR-pMHC interactions, as demonstrated by the lack of Foxp3+CD4SP thymocytes in Aβb-/- mice which lack MHC class II (Fontenot et al., 2005b).

As mentioned, thymic epithelia express the transcription factor AIRE which allows promiscuous expression of tissue-specific antigens in the thymus (Anderson et al., 2002) and has been suggested to play a role in selection of Foxp3+ Tregs (Aschenbrenner et al., 2007). However, the role of AIRE has been mostly attributed to the deletion of auto-reactive T cells rather than selection of Tregs. Some groups have produced data that supports the model that increased affinity for self-peptide;MHC complexes favours Treg development (Jordan et al., 2001; Picca et al., 2006; Yu et al., 2008), proposing that Tregs show a higher affinity towards self-antigen than T effector cells. Investigations of the TCR repertoires of CD25+ and CD25- T cells showed that their TCRs were comparable in diversity, but distinct and only showing partial overlap (Hsieh et al., 2006; Pacholczyk et al., 2007; Wong et al., 2007). Although one of these studies concluded that Tregs were reactive to non-self rather than self (Pacholczyk et al., 2007) it is believed that most Tregs show an inherent self-reactivity and this has been proposed to be a positive factor in peripheral maintenance of Tregs.

Compared to CD25- cells, CD25+ Tregs show increased proliferation in the steady state in vivo, proposed to be through constant low level TCR stimulation by self antigens in the periphery (Fisson et al., 2003). Furthermore, retroviral transduction of TCRs cloned from CD4+CD25+ Tregs, but not CD4+CD25- cells, resulted in T cells which underwent massive expansion upon transfer to a lymphopaenic host (Hsieh et al., 2004). Although a lymphopaenic environment is not the most physiologically relevant situation,

this enhanced proliferation of cells transduced with Treg TCRs was proposed to be due to the reactivity of these TCRs against self-peptides.

1.2.5 Signals which drive Treg selection

1.2.5.1 TCR signals

Some of the studies mentioned in section 1.2.4 proposed a model of instructive Treg generation, whereby self-antigen specifically drives a cell towards a Treg fate (Jordan et al., 2001; Yu et al., 2008). However, other studies proposed a more selective model where commitment to the Treg fate is random, but Treg populations show enhanced resistance to deletion compared to non-Tregs. Self-reactive Tregs are not, therefore, deleted in the thymus and thus skews their TCR repertoire towards self-reactivity (van Santen et al., 2004). A recent study by Lio et al (Lio and Hsieh, 2008) has suggested the thymic development of Tregs is an instructive mechanism divided into two processes; 1) TCR-mediated self-peptide recognition, driving up-regulation of CD25 and 2) TCR-independent cytokine signals that drive Foxp3 expression and commitment to the Treg lineage. The requirement for TCR-mediated signaling events in Treg thymic generation was emphasised in mice which have a defect in a major TCR signaling component linker for activated T cells (LAT) (Koonpaew et al., 2006). The mutation prevents binding to the downstream signaling molecule PLCγ1 and these mice have a severe deficit in both thymic and peripheral Foxp3+ cells. Importantly, LAT-defective mice showed no defects in positive selection emphasising that this process is distinct from the signals that drive selection towards the Treg lineage.

1.2.5.2 IL-2

Deficiency of IL-2 or the components of its receptor, CD25 (IL-2Rα) and CD122 (IL-2Rβ), result in fatal lympho-proliferative disease in mice (Horak et al., 1995) and similar

deficiencies have been reported in humans (Sharfe et al., 1997). These disorders were proposed to be due to deficiencies in either the thymic development or peripheral maintenance CD25+ Tregs (Bayer et al., 2005; Furtado et al., 2002; Malek et al., 2002). Using CD4+ cells from IL-2-/- mice, Furtado et al could transfer protection from autoimmunity to WT hosts, but this could not be seen with CD25-/- donor cells (Furtado et al., 2002). They reasoned that in the absence of IL-2 the thymic development of Tregs could occur, but that IL-2 was essential for their suppressive function in the periphery. With the use of Foxp3-GFP mice it was confirmed that mice deficient in either IL-2 or CD25 had normal induction of Foxp3 in thymocytes (Fontenot et al., 2005a). However, this group also identified that the deficiency in Tregs was exacerbated in mice lacking the common γ-chain receptor, suggesting that in the absence of IL-2, IL-15 may compensate, thus some degree of signalling via the γ-chain is required for thymic generation of Tregs.

While many have suggested that IL-2 is essential for Treg function, some studies suggest that it is dispensable for Treg-mediated suppression (D'Cruz and Klein, 2005). This study argued that IL-2 was only necessary for the maintenance of Tregs in the periphery, although they could not rule out the possibility that IL-2 may be a maturation signal that allows Treg emigration from the thymus.

1.2.5.3 Downstream signals of IL-2

Signalling via the common γ-chain activates signalling molecules, janus kinases (Jaks). Like the γ-chain deficient mice, Jak-/- mice also lack thymic and peripheral Foxp3 expression (Mayack and Berg, 2006). IL-2-mediated Jak activation leads to the phosphorylation of members of the Stat family (Signal transducers and activators of transcription), Stat5a and Stat5b (Johnston et al., 1995). Investigating the role of Stat5a and b, Yao et al. (Yao et al., 2007) demonstrated that these signals occurring downstream of IL-2 signalling were essential and non-redundant for Treg cell function

and for optimal Foxp3 induction. Foxp3 was shown to be a direct target of Stat5 as STAT-5 binds to the Foxp3-promoter (Burchill et al., 2007). More recently a study demonstrated that constitutive STAT-5 signalling could overcome the deficiency of Tregs in CD28-/- mice and drive T cells that would normally become effector T cells towards a regulatory phenotype towards a regulatory cell lineage (Burchill et al., 2008).

1.2.5.4 CD28

CD28 co-stimulation forms part of signal 2 in the T cell activation cascade and is essential for full T cell function. NOD mice deficient in CD28 signalling were shown to develop accelerated diabetes and to lack peripheral Tregs. This was proposed to be due to the lack of IL-2 from activated Teff cells and therefore Tregs could not be maintained (Salomon et al., 2000). However, thymocytes in CD28-/- mice also did not express Foxp3 (Tai et al., 2005). CD28 signalling in thymocytes was shown to directly drive Foxp3 expression, independently of IL-2 signalling (Tai et al., 2005); however, while these cells expressed GITR and CTLA-4, they did not up-regulate CD25 or show suppressive capacity, therefore were described as immature thymic Tregs.

A recent article by Burchill et al combines the pathways mentioned above and suggests that the TCR/CD28 and cytokine signals together drive the development of regulatory T cells (Burchill et al., 2008). Their data present a model where TCR/CD28 signalling initiates the pathway towards the Treg lineage, including up-regulation of Treg markers and cytokine receptors, followed by the production of cytokines that drive the activation of STAT-5 and stabilises the Treg phenotype.

1.2.6 Activity of Foxp3.

Foxp3 is a member of the forkhead/winged-helix family of transcriptional regulators which possess three functional domains (Figure IIIA) (Campbell and Ziegler, 2007). Mutations in these domains have begun to reveal the functions of each (Lopes et al., 2006; Marson et al., 2007; Wu et al., 2006). The forkhead domain (FKH) acts as a nuclear localisation factor and DNA-binding domain (Schubert et al., 2001). NFAT (Nuclear factor of activated T cells) proteins play a major role in the transcription of key genes in T cell activation, including many cytokine genes. NFAT binds co-operatively to transcriptional co-factors, notably AP-1, which allows binding to composite sites in the regulatory regions of their target genes to initiate transcription (for a review on NFAT and its function, see (Rao et al., 1997)). FKH binding sequences have been identified next to NFAT regulatory sites in the promoters of cytokine genes (Schubert et al., 2001). Foxp3 was shown to repress NFAT-mediated transcription, suggested to be via competition with NFAT (Schubert et al., 2001). Later work showed that suppression of transcription was associated with direct Foxp3-NFAT interactions (Bettelli et al., 2005).

More recently, Wu et al. (Wu et al., 2006) determined that Foxp3 directly interacts with NFAT. Using guided mutations determined by the crystal structure of the protein interactions the investigators could interfere with the Foxp3-NFAT binding in a graded fashion. Progressive disruption of the interaction affected the ability of Foxp3 to repress il-2 transcription in a graded manner. This study suggested that NFAT can drive both immunity and tolerance in T cells via differential recruitment of the transcription factors AP-1 and Foxp3 respectively (Figure IIIB) (Wu et al., 2006). Following on from this observation, it was shown that the N-terminus of Foxp3 inhibits the promoter activity of AP-1 by binding to phosphorylated sites and altering its sub-nuclear localisation, suggesting a mechanism of how Foxp3 may compete against NFAT-AP1 binding (Lee et al., 2008a).

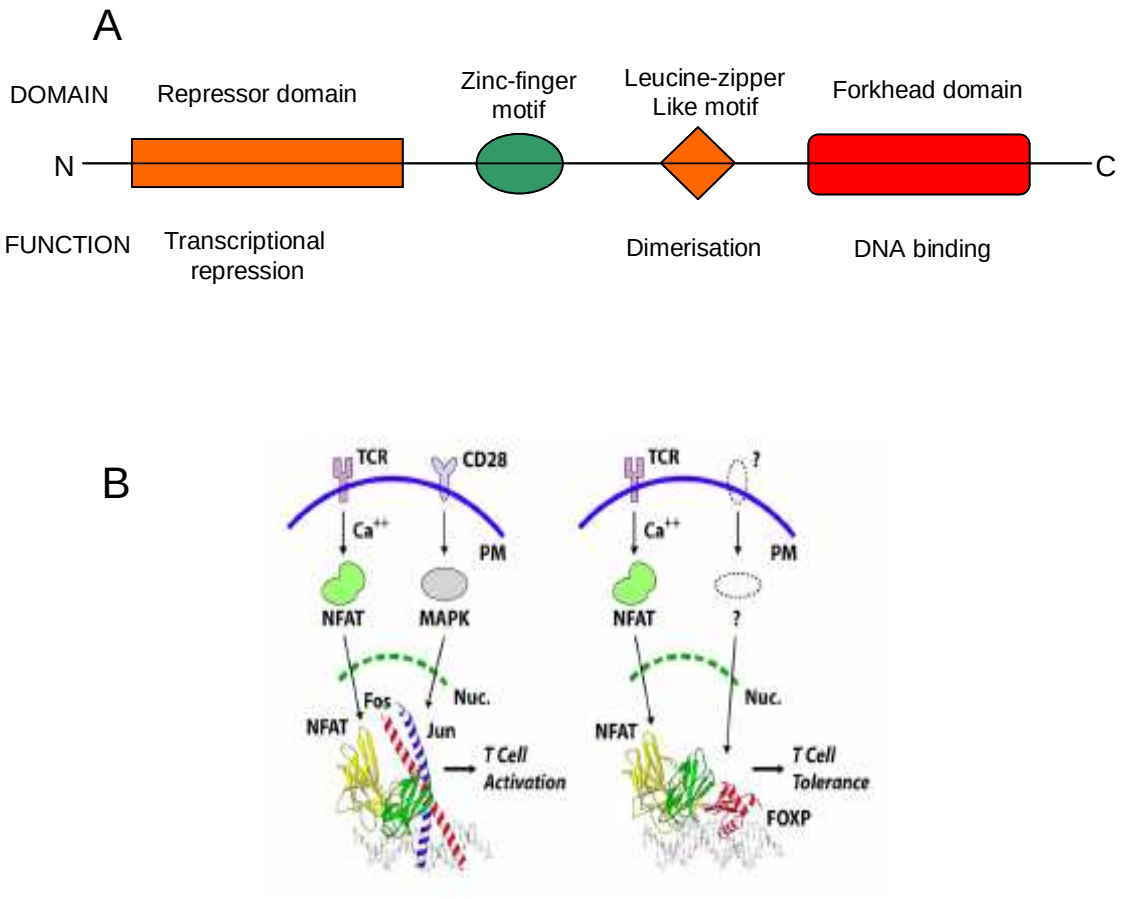


Figure III: Structure and function of FOXP3. A: Schematic diagram of the FOXP3 showing individual domains and a description of their function as discovered by site directed mutagenesis (Adapted from Campbell and Zeigler Nat. Rev. Imm. 2007). B: Left: Schematic representation of the T cell activation program mediated by TCR/CD28 signals involving the NFAT:Fos:Jun complex. Right: In regulatory T cells TCR signalling may be affected by an as yet unknown signal that drives the co-operation of FOXP3 with NFAT and its interaction with composite DNA elements. The NFAT:FOXP3:DNA complex regulates the expression of genes required for the development and function of Tregs. (Adapted from Wu. et al. Cell 2006).

To determine the target genes of Foxp3 two groups have used chromatin-immunoprecipitation (ChIP) and DNA microarray techniques to identify the promoter regions bound by Foxp3. One group, using FLAG-tagged Foxp3-transduced CD4+ T cell hybridomas, identified ~1,100 genes regulated by Foxp3 (Marson et al., 2007). The other group used primary CD4+ T cell cultures and identified ~700 genes targeted by Foxp3 (Zheng et al., 2007b). Discrepancies in the target gene number may be attributable to the different cell types analysed, however, one similar observation from both groups is that while Foxp3 acts as a negative regulator of many factors, such as IL-2, it also induced upregulation of other genes, encoding CD25, CTLA-4 and GITR (Marson et al., 2007; Zheng et al., 2007b) suggesting Foxp3 can also act as a positive regulator of some genes.

1.2.7 Foxp3 – essential to the Treg lineage?

The experiments involving ectopic expression, or over-expression, of Foxp3 in T cells showed conversion of naïve T cells towards a Treg phenotype (see section 2.3). These observations led to the hypothesis that Foxp3 acts as a Treg lineage specification factor. However, it was not clear if Foxp3 expression occurs as a consequence of developing thymocytes becoming committed towards the Treg lineage, or if Foxp3 initiates the commitment towards the Treg lineage. Lin et al. (Lin et al., 2007) addressed this question by using a Foxp3 allele which directs the expression of a non-functional fusion protein of Foxp3 and enhanced GFP (Foxp3ΔGFP). This fusion protein generates a Foxp3 lacking the C-terminus therefore cannot translocate to the nucleus to bind NFAT. Mutant Foxp3ΔGFP mice showed normal thymocyte development, similar to wild-type mice, and mutant thymic GFP+ cells expressed the Treg markers CD25, CD62L, CTLA-4 and GITR. This study suggested that Treg thymic generation could occur in the absence of Foxp3 and that Foxp3 was not responsible for driving the Treg lineage.

In confirmation with this study, the majority of signature Treg-associated genes were shown not to be due to Foxp3 expression, but were genes that could be co-regulated with Foxp3 (Hill et al., 2007). These studies highlighted that signals up-stream of Foxp3 expression must drive T cells towards a Treg lineage. However, the Foxp3- mutant cells could not suppress the anti-CD3 induced proliferation of naïve splenocytes, suggesting that nuclear localisation is required for suppressive function of the Tregs (Lin et al., 2007). Foxp3ΔGFP thymocytes showed a four-fold increase in the number of late apoptotic cells, consistent with the notion that Foxp3+ Tregs have an intrinsic resistance to apoptosis and enhanced survival compared to Foxp3- cells (Papiernik et al., 1998; Taylor et al., 2007).

The requirement for Foxp3 expression to maintain Treg function in the periphery was demonstrated by Williams et al. (Williams and Rudensky, 2007) using a loxp-flanked Foxp3 allele. When Foxp3 was removed from these cells they lost suppressor cell function and, interestingly, gained the ability to produce IL-2 and effector cytokines (Williams and Rudensky, 2007).

1.2.8 Other regulatory cell types

1.2.8.1 Tr1 cells

T regulatory type 1 cells (Tr1) were described the Roncarolo group in the late 90's as a population of CD4+ T cells activated in the presence of high dose of IL-10 which produced significant amounts of IL-10, TGFβ and IL-5 (Groux et al., 1997). These cells were shown to suppress T cell proliferation in vivo and when co-transferred with CD45RBhigh T cells they could suppress IBD in SCID mice. Murine Tr1 cells do not produce IL-4 or IFNγ (Battaglia et al., 2006a) making these cells distinct from Th cells, however human Tr1 cells were shown to make IFNγ albeit at a lower level than Th1 cells (Bacchetta et al., 2002). Like nTregs, Tr1 show a very low proliferative capacity to

TCR stimulation, but can be expanded in vivo in the presence of IL-2 (Bacchetta et al., 2002). Tr1 cells also express normal levels of activation markers after activation, although these cells do not express Foxp3 and a definitive marker for Tr1 remains elusive (Battaglia et al., 2006a; Vieira et al., 2004). IL-10 is largely considered to drive Tr1 generation (Groux et al., 1997) although stimulation of T cells with vitamin D3, dexamethaosone and anti-IL-12, -IL-4 and -IFNγ antibodies have all been reported to induce Tr1 differentiation (Barrat et al., 2002). Stimulation of T cells with immature DC (iDC) also generate an IL-10-producing population which resembles the Tr1 phenotype (Wakkach et al., 2003) and these IL-10-producicng cell have been proposed to play a tolerogenic role in the steady state.

The investigation into IL-10+ Tregs began after discovery of this cell phenotype in a SCID patient displaying long-term graft tolerance (Bacchetta et al., 1994). Since their identification, Tr1 cells have been associated in modulating responses to self-antigens (Groux et al., 1997) and their function is altered in certain autoimmune pathologies, for example in rheumatoid arthritis (Yudoh et al., 2000) and diabetes (Battaglia et al., 2006b). Low dose peptide therapy has also been associated with an increase in antigen-specific IL-10+ cells for cat dander allergy (Smith and Larche, 2004). Tr1 cells constitute a regulatory population that arises in the periphery in response to antigenic stimulation in the presence of high levels of IL-10 and may play a functional role in allergic/autoimmune settings.

1.2.8.2 Th3 cells

T helper type 3 (Th3) cells are generally associated with oral tolerance, secrete large amounts of TGFβ and suppress Th1/Th2 responses (Weiner, 2001). Th3 cells have also been reported to suppress autoimmunity (Carrier et al., 2007b) but this may be through their de novo induction of Foxp3+ Tregs (Carrier et al., 2007a) as TGFβ signals have

been shown to convert CD25- T cells to Foxp3+ T cells in the periphery (Zheng et al., 2007a).

1.2.8.3 CD8+ Tregs

The initial discovery of suppressor T cells by Gershon and Kondo in the 1970's was largely focussed on CD8+ cells (Gershon and Kondo, 1970; Gershon and Kondo, 1971). Since the revival of regulatory T cells in the early 1990's, CD4+ cells have taken centre stage, however, CD8+ Tregs are now coming back into favour (Smith and Kumar, 2008) . CD8+ cells have been demonstrated to be induced to become regulatory and are dependent on the production of IFNγ, which stimulates the production of TGFβ (Myers et al., 2005) and CD8+ Tregs have been demonstrated to play a role in allograft survival (Guillonneau et al., 2007). This study identified that IFNγ production by CD8+ cells induced IDO expression by the vascular endothelial cells of the graft, thus generating an immune privileged site. A recent study has identified CD8+ regulatory T cells in MS patients (Correale and Villa, 2008). These cells show cytolytic activity against myelin-reactive CD4+ T cells and interestingly, reduced levels of CD8+ Tregs correlated with relapses in the patients. CD8+ Tregs have also been implicated in recovery from EAE, the mouse model of MS (Lee et al., 2008b).

1.2.8.4 B cell regulation

While B cells have been implicated in the pathogenesis of many chronic immune responses, including autoimmunity, more recent studies have identified a role for B cells in regulating immune responses. Triggering of naïve B cells via TLRs induces IL-10 production, while CD40 signalling has also been shown to be important (Fillatreau et al., 2008). B cells producing IL-10 have been shown to play a role in models of chronic intestinal inflammation (Mizoguchi et al., 2002), rheumatoid arthritis (Mauri et al.,

2003) and EAE (Fillatreau et al., 2002). Indeed, B cell IL-10 production was shown to be reduced in patients with MS attributed to the fact that there were fewer naïve B cells in these patients compared to healthy controls (Duddy et al., 2007). However, while B cell IL-10 has been shown to be essential in EAE (Fillatreau et al., 2002), Foxp3+ Tregs were found to be the essential source of IL-10 within the CNS (McGeachy et al., 2005).

1.2.9 Mechanisms of Regulation

In the periphery Tregs have been described to have four main functions of suppression; suppressive cytokines, cytolysis, metabolic disruption and modulation of APCs (Vignali et al., 2008). Originally, upon the discovery of Tregs it was suggested that they function in a cell-contact dependent fashion either between Tregs and APC (Takahashi et al., 1998) or between Tregs and responder cells in vitro (Thornton and Shevach, 1998). However, in vivo models have revealed behaviour of Tregs which is not seen in vitro, and may represent more physiologically relevant mechanisms of Treg-mediated suppression (Klein et al., 2003)

1.2.9.1 Cytokines

IL-10 and TGFβ are the cytokines most commonly associated with TReg-induced suppression. As mentioned above, IL-10 and TGFβ are produced by peptide-induced Tr1 and Th3 cells respectively, but thymically derived Foxp3+ Tregs are also proposed to secrete these cytokines. IL-10 was shown to be responsible for Treg suppression of intestinal inflammation, as Tregs from IL-10-/- mice or blockade of IL-10 using mAbs prevented recovery from CD4+CD45RBhi transfer-induced colitis in SCID mice (Asseman et al., 1999). Also, IL-10 has been shown to play a role in allergic and asthmatic responses (Hawrylowicz, 2005) and in autoimmunity (McGeachy et al., 2005). As mentioned above peptide-induced Tr1 cells are proposed to be a major source of IL-

10 however, both natural and induced Tregs have been shown to play a role in the resolution of some responses (Hawrylowicz, 2005). IL-10 is produced by a number of cell types, including B cells, mast cells, NK cells and DCs (Moore et al., 2001) but a recent study has targeted the specific deletion of IL-10 from Foxp3+ T cells (Rubtsov et al., 2008). By generating Foxp3YFP-cre mice crossed with il10flox/flox this group showed that Treg-derived IL-10 was not solely responsible for prevention of autoimmune responses, seen with Foxp3-/- mice, but are essential to the control of self- and environmental antigen tolerance and limiting hyper-responsiveness to antigens at mucosal surfaces.

The role of TGFβ in Treg-mediated suppression appears more contentious, with some reports suggesting it is not important in nTreg-mediated suppression (Piccirillo et al., 2002) while others have reported that CD45RBhigh T cells expressing a dominant negative TGFβ receptor could not be suppressed by Tregs in the SCID transfer model of IBD (Fahlen et al., 2005). Some in vitro studies have suggested that while soluble TGFβ does not play a role in T cell suppression, membrane bound TGFβ is required for maximal Treg suppression (Nakamura et al., 2001). Membrane bound TGFβ has also been shown to play a role in suppression of CD8+ T cells (Green et al., 2003) and the decline of membrane-bound TGFβ is associated with the loss of Treg suppression and subsequent development of autoimmune diabetes (Gregg et al., 2004).

More recently a new member of the IL-12 family, IL-35, has been proposed to play a role in Treg suppression (Collison et al., 2007). This study demonstrated that the two components of IL-35, Ebi3 (IL-27β) and Il12a (p35) are up-regulated in Foxp3+ Tregs and are boosted when Tregs are co-cultured with Teff cells. IL-35 suppressed T cell proliferation and conferred regulatory activity when ectopically expressed in naïve T cells (Collison et al., 2007). IL-35 has also been shown to be effective at suppressing Th17 cells and can function therapeutically in collagen-induced arthritis model (Niedbala et al., 2007). Further investigation into this cytokine will determine if it is

indeed a Foxp3+ Treg specific cytokine and the role it may play in Treg induction/expansion and regulation.

1.2.9.2 Cytolysis

Release of granzymes as mediators of virally-infected or tumour cell elimination has long been associated with the activity of CD8+ T cells and NK cells (Lieberman, 2003). However, investigation into the genes up-regulated by Foxp3+ Tregs revealed the expression of granzyme B in these cells (Zheng et al., 2007b). Gondek et al. have also demonstrated that Tregs do possess cytolytic activity in a granzyme B-dependent fashion in vitro (Gondek et al., 2005) and this was later confirmed to be required for Treg suppression of B cells in vivo (Zhao et al., 2006). Other granzyme-independent pathways of apoptosis have also been suggested, for example via galectin-1 (Garin et al., 2007) and TRAIL (Ren et al., 2007). While Treg killing has been demonstrated, the importance of this mechanism in suppression is yet to be fully elucidated and it would be difficult to explain bystander suppression mechanism in this context, as direct cell contact would be required for this function of Tregs.

1.2.9.3 Metabolic disruption

As Tregs constitutively express the high affinity IL-2 receptor acting as an IL-2 ‘sink’ was one of the intital hypothesis of Treg function (Sakaguchi et al., 1995) and was shown to be an effective mechanism in vitro (de la Rosa et al., 2004; Scheffold et al., 2007). Recently Treg-mediated cytokine deprivation and apoptosis of Teff cells was also shown to function in vivo in a mouse model of IBD (Pandiyan et al., 2007).

1.2.9.4 Effects on APC

While suppression of T cell responses has been shown to occur in vitro in the absence of APC (Shevach, 2002) it is clear that Treg-APC interactions also occur. This has predominantly been shown via CTLA-4-B7 interactions (Takahashi et al., 2000) that have been proposed to increase IDO expression by DCs (Fallarino et al., 2003). Two studies which utilised two-photon laser scanning cytometry have been able to visualise Treg function in vivo (Chappert et al., 2008; Tadokoro et al., 2006). These studies have shown that Tregs can form stable contacts with DC, after which the DC do not form stable interactions with Teff cells. Recently, Sarris et al described selective expression of Neuropilin-1 on Tregs that enhances their interaction with DC in vitro and may give them a selective binding advantage over Teff (Sarris et al., 2008). The exact mechanisms involved in the in vivo models has not been fully examined yet, but they suggest that Treg may induce DC release of soluble mediators which prevent Teffector cell interaction or that Treg can directly prevent the activation of DC. Consistent with the later hypothesis, some studies have demonstrated that CTLA-4-B7 interactions induced down-regulation of co-stimulatory molecules on the surface of immature DC (Oderup et al., 2006).

1.2.10 Overcoming Treg suppression

Obviously in some settings a robust immune response is required and the suppression mechanisms of Tregs must be turned off. As previously mentioned, GITR ligation on both Tregs and Teff cells has been shown to abrogate suppression, although this has been attributed to GITR signalling in the responder T cells rather than in Tregs (Stephens et al., 2004). Other cell surface molecules such as OX40 (Valzasina et al., 2005) and 4-1BB (Choi et al., 2004) have also been proposed to play a role in negative stimulation to Tregs and blocking their suppression.

Certain cytokines, such as IL-6 and IL-12 can act to release Teff cells from Treg mediated suppression (Dominitzki et al., 2007; Fehervari and Sakaguchi, 2004). Importantly, these cytokines are released from activated DC, usually via TLR stimulation. TLR expression has also been detected on Tregs (Caramalho et al., 2003). Two recent studies have investigated the effect of TLR2 ligation on Tregs. Combined with IL-2 and TCR stimulation, ligation of TLR2 induced Treg proliferation and abrogated their suppressive capacity (Kabelitz, 2007; Liu et al., 2006). Foxp3 expression was also shown to be reduced following TLR2 stimulation (Liu et al., 2006) suggesting a possible, undefined mechanism for controlling suppression.

Tregs clearly function by a number of different mechanisms, proposed by Vignalli – how many mechanisms do Tregs need? (Vignali et al., 2008). It is likely that the varied mechanisms of Tregs represent the many functions they play in our immune system. Depletion of some mechanisms of suppression results in T cell hyper-activation and autoimmunity, while elimination of other does not result in autoimmunity, but in the activation of T cells at mucosal surfaces. These differences probably reflect the multiple roles that Tregs play, from maintaining peripheral homesostasis, to preventing excessive damage during inflammation and maintaining potentially auto-aggressive lymphocytes in the periphery in a dormant state. These functions may be controlled by different subsets of Tregs including both natural and induced Tregs, but also sub-division between these groups based on their integrin and chemokine receptor expression. Ultimately, the balance between these cells and effector T cells determine the outcome of our immune response in both the steady state and during infection, for better or worse.

1.3 Multiple Sclerosis and EAE

Multiple Sclerosis (MS) is a chronic inflammatory neurological disease, characterised by infiltration of mononuclear cells in the central nervous system (Noseworthy et al., 2000). Clinically, the effects of the inflammation result in plaques in the CNS that is visible via magnetic reasonance imaging (MRI) (Napoli and Bakshi, 2005). The plaques represent areas of destroyed myelin, an important component which insulates axons and allow the effective transmission of nerve impulses to and from the brain. The damage to myelin prevents signals to the muscles, resulting in muscle weakening and progressive disability in many patients (Trapp et al., 1999). MS usually begins in young adulthood and can follow a primary-progressive course or relapsing-remitting course, where between episodes of disease there is a period of time where no disease is apparent (Noseworthy et al., 2000). Initiation of MS, genetic and environmental factors affecting disease course and the cellular components which propagate the disease have been investigated as extensively as possible in humans. However, much of our understanding has come from development of a mouse model of MS, EAE. Encephalomyelitis was first identified by Rivers et al. after injection of attenuated rabies vaccines grown in rabbit brain. Brain lesions that were observed after vaccination were suggested to be due to antigens in the rabbit brain tissue Active induction of encephalomyelitis was shown to be induced in animals using CNS-derived antigens emulsified in complete Freunds adjuvant (CFA) and formed the basis of the EAE model (Kabat et al., 1946). EAE is therefore an induced model of disease and while it has obvious differences from MS, it provides an effective immunological tool to study the basis of disease mechanisms and highlight potential targets for translational therapy.

1.3.1 Susceptibility to MS

There is strong evidence to suggest that environmental and genetic factors play major roles in susceptibility to MS (Sotgiu et al., 2004). The geographical clustering of high incidence of MS strongly suggests environmental factors can enhance the risk of developing MS. These factors include exposure to certain infectious oragnsims (Ascherio and Munger, 2007a), but also non-infectious parameters such as exposure to sunlight (Ascherio and Munger, 2007b).

Genetic linkage to MS susceptibility has long been suggested (Jersild et al., 1973) and confirmed with recent investigations into the genes that are associated with disease susceptibility. Of the genes associated with MS, the strongest genetic association is with the MHC complex. Certain human leukocyte antigens (HLA) have shown the strongest correlation with MS, particularly HLA-DR2 (Etzensperger et al., 2008). Transgenic mice were generated that expressed the HLA-DR2 element DRB1*1501 and that expressed a TCR from patients with MS (Madsen et al., 1999). These mice developed clinical signs of paralysis and confirmed that expression of particular MHC genes will affect susceptibility to MS.

More recently using DNA microarray technology, alleles of IL2RA and IL-7RA were confirmed along with alleles of the HLA locus as heritable risk factors for MS (Hafler et al., 2007). As the IL-2Rα chain (CD25) is expressed by the majority Tregs (Sakaguchi et al., 1995) and the recently described role for IL-7 in the generation of Foxp3+ Tregs (Bayer et al., 2008; Mazzucchelli et al., 2008) the effect of these risk alleles on Treg development and function in MS patients is of great interest.

1.3.2 MS as an immune-mediated disease

The damage of the myelin sheath in MS is largely believed to be the result of a autoimmune response, mediated by self-reactive lymphocytes (McFarland and Martin, 2007). The role of the immune response in MS is highlighted by the efficacy of therapeutic targets of MS and EAE which almost exclusively target at least one aspect of the immune response. Monoclonal antibody therapies that deplete immune cells (Brostoff and Mason, 1984) or that target immune cell activation and migration to the CNS (Linker et al., 2008) have been shown to stop inflammation.

B cell-specific depletion using the mAb Rituximab (anti-CD20) reduced inflammatory lesions in the brain (Hauser et al., 2008). Further contributing to the evidence for a role of B cells in MS, anti-myelin antibodies can be detected in the cerebro-spinal fluid of patients after a primary MS event (Berger et al., 2003).

T cells have also been identified in the lesions of chronic MS lesions (Traugott et al., 1983) and T cells isolated from peripheral blood of MS patients are reactive against a number of CNS components, including myelin basic protein (MBP), proteo-lipid protein (PLP), myelin ologodendrocyte glycoprotein (MOG) and stress proteins such as αB-βcrystallin (Steinman, 2001). Although CD4+ and CD8+ neuroantigen-specific T cells can be found in both healthy people and MS patients, the T cells in MS patients have an altered phenotype, suggesting differential activation (Crawford et al., 2004). While CD8+ T cells have been suggested to proliferate more extensively than CD4+ cells in MS patients (Babbe et al., 2000), perhaps the largest indicator that CD4+ T cells are involved in MS is the association of HLA (MHC) class II molecules with heightened susceptibility to disease (Olsson et al., 2006). How MHC background can affect autoimmunity is discussed in the next section.

Autoreactive CD4+ T cells which can proliferate in response to MBP and PLP have been identified in the peripheral blood and CSF of MS patients (Zhang et al., 1994). A

more recent study has identified a correlation between the presence of T cells and antibodies in the blood which react to myelin epitopes and the formation of lesions in the brain (Greer et al., 2008). These studies strongly suggest that adaptive immune cells play an important role in the pathogenesis of MS and support the classification of MS as an immune mediated disease.

Treatment with IFNβ is an approved therapy for MS, and is associated with a reduction in inflammatory lesions (de Andres et al., 2007; Li et al., 2001). Treatment is associated with closure of the blood-brain barrier (BBB) and down-regulation of VLA-4 on CD4+ T cells (Calabresi et al., 1997; Stone et al., 1995). Human trials of an anti-VLA-4 mAb have shown reductions in inflammatory lesions and reduced relapse rates (Miller et al., 2003). TNFα and IFNγ induce up-regulation of VCAM on endothelial cells and increased VLA-4 on CD4+ T cells, thus facilitating their migration across the BBB. However, to migrate through the extracellular matrix, matrix metallo-proteinases (MMP) are required to degrade the collagen and allow lymphocyte access. MMPs are detected in the CSF of MS patients and are secreted from endothelial cells, macrophages and astrocytes of the lesions and myelin-specific T cells have been shown to produce MMPs upon activation (Conlon et al., 1999).

Other cell types, including CNS-resident cells and cytokines have also been shown to be involved in the destruction of the myelin sheath. Steinman (Steinman, 2001) has proposed a two-stage model of MS whereby neuroantigen-reactive T cells become activated (see later discussion) and induce inflammatory cytokine release, including IFNγ and TNFα which can allow their migration across the BBB by up-regulation of adhesion molecules. Secondarily, activation of macrophages, microglial cells (Benveniste, 1997) and astrocytes in the CNS by lymphotoxin-α (LTα) and TNFα induces the up-regulation of iNOS, which catalyses nitric oxide (NO) synthesis, and mediates killing of oligodendroglial cells. Many cells, both immune and non-immune, can therefore contribute to MS pathogenesis, however from these studies it is likely that an immune-mediated mechanism is ultimately responsible for development of disease.

1.3.3 EAE as an immune mediated disease

Like MS, macrophages play a major role in the initiation of the EAE by release of cytokines, such as LTα and TNFα and IL-12 (Bauer et al., 1995; Benveniste, 1997). These upregulate co-stimulatory molecule expression and enhance antigen presentation on microglia and astrocytes and allow permeability of the CNS to other leukocytes. The pathogenesis of EAE is mediated by CD4+ T cells, demonstrated by the appearance of clinical disease upon transfer of CD4+ cells purified from mice with EAE (Zamvil et al., 1985) and the requirement of CD4+ T cell activation for disease (Grewal et al., 1996). Furthermore, EAE cannot be induced when CD4+ T cells are depleted (Brostoff and Mason, 1984).

Originally EAE was described as a Th1 mediated disease, however it has been documented that IFNγ-/- mice still develop EAE, even suggesting IFNγ has a regulatory role in EAE as these mice have a more severe phenotype than IFNγ-sufficient mice (Ferber et al., 1996). With the discovery of the Th17 subset many models with Th1 origins are now being re-assessed as Th17 mediated diseases, including EAE. This discovery began with the finding that mice deficient in the IL-12 subunit, p35, were susceptible to EAE, while IL-12 p40-/- mice were resistant (Cua et al., 2003; Gran et al., 2002). As mentioned, IL-23 was discovered to be composed of the p19 and the IL-12p40 subunits (Oppmann et al., 2000), the role of IL-23 in driving the Th17 subset was identified and reported to be the mediator of the CNS inflammation (Langrish et al., 2005). Investigations into the Th17/Th1 mechanisms of CNS inflammation represent an extensive field of current immunology and while some studies have discredited Th1 cells from pathogenesis, others studies have found that IFNγ and Th1 populations are still involved in EAE (Kroenke and Segal, 2007; O'Connor et al., 2008; Thakker et al., 2007).

1.3.4 Antigenic targets for CNS-reactive lymphocytes

The target antigens known to be involved in MS and EAE are components of the myelin sheath. PLP compromises more than 50% of myelin protein and was identified as a target for autoimmune cells in EAE using synthetic PLP peptides (Tuohy et al., 1989; Williams et al., 1982). MBP also contributes to a large proportion of myelin protein (~30%) and has been shown to have encephalitogenic potential (Einstein et al., 1962). MOG constitutes less than 0.05% of total myelin protein, however it has been shown to be encaphalitogenic in mice, including in the C57BL/6 model (Mendel et al., 1995), as is used in this study. The pathogenic potential of MOG has been attributed to its accessibility to the immune system compared to MPB and PLP (Johns and Bernard, 1999).

The encephalitogenic region of MBP was first discovered in experiments that used synthetic peptides to induce EAE in guinea pigs (Eylar et al., 1970). Over a decade later the residues of MBP that can induce EAE in mice of the H-2^u haplotype was shown to be the n-terminus Ac1-9 epitopes (Zamvil et al., 1986). Residues 80-100 of MBP also show encephalitogenic activity in different strains of mice and were also identified as targets in human disease (Kono et al., 1988; Pette et al., 1990). Later, the encephalitogenic region of PLP was found to be PLP139-151 in mice of the H-2^s haplotype, specifically in the SJL strain of mice (Tuohy et al., 1989). The encephalitogenic region of MOG, MOG35-55, was identified in C57BL/6 mice in 1995 (Mendel et al., 1995).

1.3.5 TCR transgenic models in EAE

The use of TCR transgenic mice in EAE has allowed investigation of myelin reactive T cells in a system where antigen-specific cell numbers would normally be limited. TCR transgenic mice are generated by the expression of TCR Vα and Vβ genes that were

found to be used by encephalitogenic T cell lines that were isolated from mice that had been immunised with the antigen of interest.

In the case of MBP-reactive TCR transgenic mice, four different strains were developed. All found that the n-terminal acetylated residues were responsible for disease in mice of the H-2^u haplotype; Goverman and colleagues (Goverman et al., 1993) and Tonegawa and colleagues (Lafaille et al., 1994) produced transgenic mice that recognised the Ac(1-11) epitope of MBP, while Janeway and colleagues (Hardardottir et al., 1995) generated a TCR transgenic that recognised Ac(1-16) of MBP. Tg3 and Tg4 mice were generated by Liu et al. after demonstrating that MBP-reactive T cell clones isolated from B10.PL mice (H-2^u) express the Vβ8.2 chain and recognised the Ac(1-9) residues of MBP (Liu et al., 1995). Tg4 mice require immunisation with peptide to induce EAE and rarely develop EAE spontaneously. In this thesis, Tg4 mice are used extensively as the MBP(Ac1-9)-reactive TCR transgenic model to investigate Foxp3+ Tregs in EAE.

The transgenic mouse relevant to the 35-55 epitope of MOG is the 2D2 mouse strain, developed by Betelli et al (Bettelli et al., 2003). The TCR in this model utilise the Vα3.2 and Vβ11 gene segments and are expressed on the C57BL/6 (H-2^b) background.

PLP-reactive TCR transgenic mice were also generated from T cell clones derived from PLP-immunised SJL mice (Waldner et al., 2000). The T cells in these mice express Vα4+Vβ6+ TCRs and respond to PLP(139-151) presented by cells from mice on the H-2^s background. However, PLP(139-151)-reactive transgenic mice frequently developed spontaneous EAE and could not be maintained on the SJL background.

1.3.6 Breach of tolerance to CNS antigens

1.3.6.1 CNS antigen expression in the thymus

The CNS was largely believed to be devoid of immune infiltrates and proposed to be a site of immune privilege (Barker and Billingham, 1977). However, the CNS has now been shown to be immune competent (Ransohoff et al., 2003) therefore other mechanism of tolerance must exist to prevent CNS-reactive T cell activation. One such mechanism that has been proposed is in situ CNS-mediated tolerance of myelin-reactive T cells (Brabb et al., 2000). Some of the CNS self-antigens which are relevant for MS pathogenesis are expressed in the thymus. For example, MBP is expressed in the mouse thymus and increased thymic expression is associated with higher resistance to MBP-induced EAE (Liu et al., 2001). MBP-reactive T cells however are still present in the periphery of mice and humans.

In the thymus, MBP shows dominant expression of one epitope due to the activity of specific protesases, including Asparagine Endopeptidase (AEP) (Antoniou et al., 2000; Manoury et al., 1998). AEP has been suggested to cut MBP at the asparagine at position 94 (Manoury et al., 2002). Alterations in the activity of AEP has therefore been suggested to alter the availability of normally cryptic eptiopes that the immune system may encounter and potentially cause autoimmunity (Anderton et al., 2002). Using the Tg4 MBP(Ac1-9) transgenic, Liu et al. demonstrated that T cells reactive to this peptide are not deleted in the thymus (Liu et al., 1995). These studies show that expression of self-peptide in the thymus is not sufficient for central tolerance and that the peptide must be recognised by the TCR with a high enough affinity to induce deletion.

PLP is also expressed in the mouse thymus, however it is only in the form of a splice variant termed DM20, which lacks the amino acids 115-151 (Pribyl et al., 1996). SJL mice, a strain which is highly susceptible to PLP-induced EAE, possess a high frequency

of PLP139-151-reactive CD4+ T cells (Anderson et al., 2000). The absence of this epitope in the thymus prevents deletion of these autoreactive T cell from the repertoire.

MOG-expression has also been detected in the thymus using highly sensitive detection methods in both mice (Pitkanen and Peterson, 2003) and humans (Gotter et al., 2004). However, studies have suggested that expression of MOG in the thymus is insufficient at affecting the peripheral MOG-reactive repertoire (Delarasse et al., 2003; Fazilleau et al., 2006).

Thymic deletion can also be affected by MHC and the ability to present antigen. One study has demonstrated the effect of poor HLA binding in the thymus can lead to an increased frequency of myelin-specific T cells in MS patients (Bielekova et al., 2004). The T cells were biased towards recognition of epitopes with predicted low binding affinities for HLA-DR molecules. This suggests that MHC background will affect repertoire of self-antigen which can be presented in the thymus and differential binding affinity of peptides to the MHC may allow escape of auto-reactive T cells in the periphery and ultimately determine susceptibility to autoimmune diseases.

1.3.6.2 Molecular mimicry

As mentioned, T cells may recognise a molecule which expresses similar amino acid patterns to their cognate peptides in a process termed molecular mimicry. Viruses such as herpes-siplex virus 6, influenza, measles and Epstein-Barr virus all express genes encoding myelin-like sequences (Wucherpfennig and Strominger, 1995). Human T cell clones reactive against MBP were shown to respond to viral peptides in vitro, suggesting this may be a functional process which can activate auto-aggressive T cells (Wucherpfennig and Strominger, 1995). A similar model of virus-induced disease has been shown in EAE (Gautam et al., 1998). While this mechanism of T cell activation has

been proposed in many inflammatory disorders (Wucherpfennig, 2001) a clear role for molecular mimicry in MS is lacking (Benoist and Mathis, 2001).

1.3.6.3 Defective Regulation

In EAE, CD25+Foxp3+ Tregs have been shown to accumulate and proliferate in the CNS concomitant with recovery from disease (McGeachy et al., 2005; O'Connor et al., 2007). Depletion of Tregs prevents the recovery from disease and allows activation of low affinity self-reactive T lymphocytes, therefore these cells are strongly implicated in control of the inflammatory response (McGeachy et al., 2005; Reddy et al., 2004; Stephens et al., 2005). One study has suggested that Tregs are impaired in chronic EAE, with reduced total Foxp3+ cells and reduced expression of Foxp3 mRNA (Matsumoto et al., 2007). Depletion of Tregs also abrogated the resistance of mice to undergo a secondary disease episode in response to immunogenic challenge (McGeachy and Anderton, 2005) suggesting that these cells are key to both recovery and protection from relapses. Another study demonstrated the role of Tregs in raising the threshold at which auto-reactive T cells could respond to self antigens (Stephens et al., 2005). Variant analog peptides of the MBP Ac1-9 immuno-dominant epitope that have sub-agonist activities do not normally induce disease when immunised in CFA. However if Tregs are depleted from the system using anti-CD25 antibodies (PC61), MBP-reactive T cells could respond to sub-agonist peptides and induce disease (Stephens et al., 2005).

No quantitative defects in Treg have been detected in the blood of MS patients compared to healthy controls; however the function of the Tregs in MS is impaired. This was shown in one study of relapsing-remitting MS (RR-MS) to be correlated with a reduction in Foxp3-expression on a per-cell basis (Venken et al., 2008b). This same group also identified that in contrast to RR-MS, secondary-progressive MS (SP-MS) did not have Treg alterations and suggested that naïve Treg vs memory Treg homeostasis may different in these two types of the disease (Venken et al., 2008a; Venken et al.,

2006). These studies suggest that differential defects in Treg function may be associated with differences in MS etiology. Interestingly, a study into the therapeutic effects of IFNβ-1α therapy demonstrated a positive effect on the suppressive activity of Tregs from MS patients (de Andres et al., 2007). However, wether Treg dysfunction has a causal role in autoimmunity is not yet clear (Baecher-Allan and Hafler, 2004). The results of these studies would imply that although the Tregs in MS patients are defective, there are therapeutic targets that can allow effective Treg function and disease regression.

1.3.7 Targeting regulation for therapy in MS and EAE

Identification of Tregs as a key mediator of disease prevention and resolution in EAE resulted in the targeting of these cells for therapeutic intervention strategies.

1.3.7.1 Oral and Nasal tolerance

T cells that encounter antigen in a soluble form and in the absence of any 'danger signals' undergo abortive T cell activation and results in un-responsiveness to future antigen encounters – termed anergy (Schwartz, 2003). Oral tolerance describes the phenomenon of induced T cell tolerance to antigens which are fed and enter the body via the gut mucosa and gut associated lymphoid tissue, GALT. Feeding of antigens mimics the tolerance we continuously develop to commensal bacteria and food and has routinely been used in experimental settings (MacDonald, 1998). The outcome of oral tolerance depends on the dose of antigen used (Weiner, 1997). High dose antigen results in clonal deletion of antigen-reactive T cells, while low dose results in active suppression, largely by the induction of TGFβ-secreting Th3 cells (Weiner, 2001). The dose effect in oral tolerance has been investigated in an MBP-reactive TCR transgenic mouse model (Chen et al., 1996). Mice which were fed high dose MBP showed deletion or anergy of the

MBP-reactive T cells. However, low dose antigen resulted in T cells which produced IL-4, IL-10 and TGFβ. These cells protected mice from MBP-induced EAE when transferred prior to disease induction. The feeding of low dose MBP also prevented EAE induced directly in transgenic mice.

Another study investigated intranasal administration of MBP in the Tg4 model (Burkhart et al., 1999). Similarly, this model demonstrated high dose antigen-induced deletion of the MBP-reactive T cells, although deletion was incomplete and the remaining cells showed hyper-responsiveness to re-stimulation with MBP. However, repeated administration effectively tolerised the transgenic T cells and correlated with increased IL-10 production. Importantly, while the transgenic mice required repeated doses of MBP to prevent EAE, non-transgenic mice only required a single i.n. dose, demonstrating that the outcome of tolerogenic protocols depends on the precursor frequency of antigen-reactive T cells (Burkhart et al., 1999). This mechanism of tolerance was shown to be most effective when using a high affinity MHC peptide analogue of MBP, MBP(Ac1-9)4Tyr. This peptide binds with a higher affinity to the MHC compared to WT MBP(Ac1-9) and therefore suggests that antigen presentation is central to the tolerance induction event.

Later investigations found that intranasally-induced tolerance to MBP was mediated by the generation of IL-10 producing CD4+ regulatory T cells, which were largely CD25-CTLA-4+ and lacked Foxp3 expression (Sundstedt et al., 2003). IL-10 and TGFβ have been implicated in the orally administered peptide-induced protection from EAE (Faria et al., 2003), however a later study demonstrated oral tolerance in IL-10-/- mice, attributing the role of IL-10 to the reduction of Th1 mediated IFNγ production (Gonnella et al., 2004). In MS, clinical trials using oral antigen administration has been relatively unsuccessful (Kappos et al., 2000; Weiner et al., 1993).

1.3.7.2 Transfer of regulatory T cells

Adoptive transfer of CD4+ T cells into transgenic mice reactive to MBP on a RAG-deficient background effectively prevented the mice from developing spontaneous EAE (Van de Keere and Tonegawa, 1998). Since realising the importance of Tregs in prevention and recovery from EAE, the direct transfer of Tregs to prevent disease has continued. One obstacle which makes Treg therapy a difficult prospect is the low frequencies of Tregs in the total lymphocyte population. This is further complicated by the much reduced frequency of antigen-relevant Tregs. Work from the Bluestone group demonstrated that Tregs could be isolated from mice and expanded in vitro using a combination of anti-CD3, anti-CD28 plus high levels of exogenous IL-2 (Tang et al., 2004b). Importantly they demonstrated that these antigen-specific Tregs were suppressive both in vitro and in vivo, with low numbers of expanded Tregs required to prevent development of Type 1 diabetes in the NOD mouse model. Kohm et al. (Kohm et al., 2002) demonstrated that transfer of high numbers of polyclonal CD4+CD25+CD62Lhi Tregs isolated from the LNs of C57BL/6 mice resulted in significant suppression of both active and passive MOG-induced EAE. Protection was associated with an increase in Th2 cytokines, namely IL-4 and IL-5, suggesting immune deviation.

Other mechanisms which target expansion of polyclonal Tregs via drug administration or vaccination have shown moderate effects on EAE (Fernandez-Martin et al., 2006; Ochoa-Reparaz et al., 2007). Investigations into the ability of transferred CD4+CD25+ Tregs which are isolated from the CNS vs naïve CD4+CD25+ Tregs to suppress EAE demonstrated that Tregs isolated from the CNS, and presumably reactive against CNS components, could suppress EAE even when very low numbers of cells were transferred (McGeachy et al., 2005). These data demonstrate that antigen-relevant Tregs are more potent than naïve or polyclonal Tregs. Generation of Treg therapies therefore requires targeting of disease-relevant Tregs to induce a population of regulatory cells which are potent enough to reduce clinical disease.

1.3.7.3 Antigen-specific Treg therapy

Expansion of antigen-specific regulatory T cells can be achieved through a number of mechanisms, one of which, oral tolerance, was mentioned previously. The von Boehmer group has shown generation of Foxp3+ Tregs in two models with the use of sub-cutaneous osmotic mini pumps (Apostolou and von Boehmer, 2004; Verginis et al., 2008). These allow the continuous infusion of low dose peptide over a period of 14 days and confer immunological tolerance to future challenge with the self-antigen.

Targeting of antigen to dendritic cells in the steady state and without activation is possible by conjugating peptide to anti-DEC-205 antibodies. This results in uptake of and presentation of the peptide without subsequent up-regulation of co-stimulatory molecules. Using this approach it has been possible to generate de novo, antigen-specific Foxp3+ regulatory T cells (Kretschmer et al., 2005). This approach, and those using the osmotic mini-pumps, confirmed that up-regulation of Foxp3 expression is associated with low levels of proliferation. Only administration of low dose antigen with the mini pumps resulted in minimal T cell proliferation and generated Foxp3+ Tregs (Apostolou and von Boehmer, 2004). Similarly, if the anti-DEC205/peptide conjugate was administered in combination with anti-CD40, the T cells proliferated extensively and no Foxp3-expression could be detected (Kretschmer et al., 2005). However, in all of the settings, induced Foxp3+ Tregs could be expanded upon immunisation with the cognate antigen.

These studies suggest that optimal generation of Foxp3+ Tregs is associated with low dose antigen administration, resulting in low level proliferation. This finding may explain the benefits of the drug Rapamycin in promoting Tregs in vitro and in vivo. Rapamycin inhibits microtubule rearrangement and therefore prevents proliferation of T cells. One study has demonstrated antigen-specific Treg induction, independent of antigen dose, by co-administration of Rapamycin (Kang et al., 2008). While this study allows the generation of antigen specific Tregs, administration of an anti-proliferative

drug systemically will also affect other cell populations limiting the use of this drug in Treg based therapies.

Understanding the mechanism behind their conversion and/or expansion is crucial to developing protocols to promote Foxp3+ Tregs. Peptide therapy therefore shows potential in inducing Foxp3+ Tregs. However, by understanding the antigenic-reactivity of disease relevant Tregs it may be possible to administer peptide alone, without the requirement of APC targeting or continuous peptide infusion. This is the ultimate aim for antigen-specific Treg based therapies in autoimmunity.

1.4 Summary

MS is a complex human disease with many potential risk factors and mechanisms of disease. It is clear that the immune system plays a key role in the inflammation associated with MS and destruction of the myelin sheath. The role of Tregs in maintaining autoreactive lymphocytes in a silent state is well documented; therefore the fact that perturbances in Treg function are associated with disease is of little surprise. By using EAE, the mouse model of MS, the function of Tregs in disease prevention, regression and remission is also clear. The disease course of some EAE models and indeed some MS cases suggests that regulation can be restored after episodes of disease; however this regulation is clearly lost, thus allowing relapses. It is likely the balance between effector, auto-aggressive T cells and Tregs will play a role. These findings suggest that restoration of Treg function and number may prevent disease development or induce disease regression. Therapeutic targeting of regulatory T cell is therefore a promising prospect for MS but requires greater understanding of the antigenic targets of Tregs and the optimum conditions which favour their conversion, expansion and potent regulatory function.

1.5 Aims of the Project

This project aimed to:

Identify the antigenic reactivity of Foxp3+ Tregs that expand during actively induced EAE (Chapter 3)

Determine the antigenic requirements for the generation of Foxp3+ Tregs in response to immunisation (Chapter 3)

Attempt to therapeutically expand disease-relevant Foxp3+ cells in situ for the prevention of EAE (Chapter 4)

From the investigations into Foxp3+ cells in naïve mice, the role of superantigen encounter on the peripheral Treg repertoire was subsequently investigated and is described in chapter 5.

2 Materials and Methods

2.1 Mice

C57BL/6, 2D2 (Bettelli et al., 2003), MOGKO (Delarasse et al., 2003), Foxp3-GFP reporter mice (Fontenot et al., 2005b) and μMT (Kitamura and Rajewsky, 1992) mice (all on the C57BL/6 background, H-2^b), B10.PL (H-2^u), Tg4 mice (Liu et al., 1995), B10.PLxSJL (H-2^{uxs}) mice were bred and maintained in specific pathogen-free conditions at the University of Edinburgh. All mice were sex-matched within experiments and used at 6-10 weeks of age, except where stated.

2.2 Antigens

2.2.1 Peptides

MOG(35-55) peptide (MEVGWYRSPFSRVVHLYRNGK), MBP(Ac1-9) peptide (ASQKRPSQR), MBP(Ac1-9) 4Tyr peptide (ASQYRPSQR) and PLP(139-151) peptide (HSLGKWLGHPDKF) were synthesized at the Advanced Biotechnology Centre (Imperial College, London).

2.2.2 Superantigens

Staphylococcal enterotoxin B (SEB) was obtained from Sigma and stored at 1mg/ml in PBS according to health and safety regulations at -80^{o}C.

2.3 General Reagents

2.3.1 Wash Buffer

RPMI 1640 medium containing 25mM HEPES buffer (Gibco, Life Technologies, Paisley, UK), 2mM L-Glutamine (Gibco), 100U/ml Penicillin, 100μg/ml streptomycin and 5x10^{-5}M 2-mercaptoethanol (all Gibco).

2.3.2 RPMI-5 tissue culture medium

Wash Buffer as above, with the addition of 5% heat-inactivated fetal calf serum (FCS) (Sigma, Poole, UK).

2.3.3 MACS Buffer

HANKS Balanced Salt Solution (Sigma), supplemented with 2% heat-inactivated FCS (Sigma) and 100U/ml Penicillin plus 100μg/ml streptomycin (both Gibco).

2.3.4 FACS Buffer

PBS supplemented with 2% heat-inactivated FCS (Sigma) and 0.05% sodium azide (Sigma)

2.4 Antibodies and FACS analysis

Cells were stained for FACS using the following antibodies (all from eBioscience, CA, except where stated). For clones see Antibody Table I.

2.4.1 Surface stains:

anti-CD4 FITC/APC/PercP (BD Pharmingen), anti-CD4 AlexaFluor700 (Caltag), anti-CD25 FITC/PE (clone 7D4, Miltenyi Biotech), anti-CD45.1 FITC/PE/biotinylated, anti-CD45.1, anti-CD90.1 FITC, anti-CD62L FITC, anti-CD44 FITC/PE, anti-CD103 FITC

2.4.2 Intracellular stains:

anti-IFNγ FITC, anti-IL-17 PE, anti-IL-10 FITC, anti-Foxp3 FITC/PE/APC staining kits and isotype control, IgG2a FITC/PE/APC, were purchased from eBioscience and used according to manufacturer' instructions. Anti-BrdU FITC/APC (BD Biosciences).
FACS data were collected on either FACSCalibur or LSR flow cytometers (BD Biosciences) and data were analysed using FlowJo software (Tree Star, CA).

2.5 Cell isolation and purifications

Peripheral lymph nodes, spleens or thymi were removed and disaggregated to single cell suspensions. Red blood cells (RBC) were lysed by incubation with Red Blood Cell Lysis Buffer (Sigma) for two minutes at room temperature (RT), followed by washing twice with MACS Buffer.

2.5.1 Preparation of mononuclear cell populations from CNS

Mice, sacrificed by CO_2 asphyxiation, were perfused with 10ml PBS through the left ventricle of the heart. Spinal cords were removed by intrathecal hydrostatic pressure using cold RPMI. Brains were removed by dissection. Spinal cord and brain were digested by mechanical disruption using a 1ml syringe then incubated for 40 minutes at 37^{o}C in 300µl of 2.5mg/ml collagenase (Worthington Biochemicals, NJ) and 1mg/ml deoxyribonuclease (DNAse) (Sigma). Cells were washed in Wash Buffer and the pellet resuspended in 30% Percoll (Gibco) (made in Wash Buffer) then underlaid with 70% Percoll. These discontinuous gradients were spun at 2000g for 20 minutes without brake. Approximately 2ml of the gradient interface was removed and washed thoroughly to obtain a single cell suspension.

2.5.2 CD4+ T cell purifications

CD4+ T cells were isolated by positive selection using CD4-conjugated MACS beads (L3T4) and MS or LS columns (all Miltenyi Biotech), according to manufacturer's instructions. Cells were incubated with 90µl MACS Buffer and 10µl CD4 Beads per 10^7 total cells for 15-20 mins at 4^{o}C. Cells were then passed through a MACS column, pre-washed with MACS Buffer attached to a MACS magnet (VarioMACS, Miltenyi Biotech). CD4+ cells were flushed from the magnet using MACS Buffer. A sample of cells was stained with a PercP-conjugated CD4+ antibody (BD Pharmingen) to assess cell purity by FACS analysis. Purity of the T cells was consistently >95%.

CD4+ cells were then either used directly for cell transfer or in vitro manipulation, or stained for further sorting on the FACSAria.

2.5.3 Regulatory T cell sorting

CD4+ cells purified by MACS as above were stained with CD4 PercP- and CD25 FITC-conjugated antibody and, in some cases, CD62L-PE conjugated antibody (all BD Pharmingen). CD4+CD25+ or CD4+CD25+CD62Lhi cells were sorted on a FACSAria (BD biosciences) to >93% purity. Expression and purity of Foxp3 was assessed by Foxp3 staining using a Foxp3-APC staining kit (eBioscience) according to manufacturer's instructions.

2.6 In vivo manipulations and antigen administration

2.6.1 Immunisations

Each mouse received 100µg pMOG, or 100µg MBP(Ac1-9 4K) or 200µg PLP(139-151) emulsified in complete Freund's adjuvant (CFA) containing 50µg of heat-killed Mycobacterium tuberculosis H37Ra (Sigma-Aldrich) at a final volume of 100µl injected sub-cutaneously into the hind legs.

2.6.2 Induction of EAE

Mice were immunised as above and also given 200ng of pertussis toxin (Health Protection Agency, Dorset, UK) in 0.5ml PBS was given intraperitoneally (i.p.) on the same day and two days after immunisation. Mice were then monitored daily for clinical signs of EAE and assessed using the following scoring system: 0, no paralysis; 1, flaccid tail; 2, impaired righting reflex; 3, partial hind limb paralyis; 4, total hind limb paralysis; 5, partial front limb paralysis; 6, moribund or dead. Mice which had lost >20% of their initial body weight or those scored as grade 5 for two consecutive days were culled. Hydrated food was provided to cages showing any signs of paralysis.

2.6.3 Peptide administration

For experiments to induce peptide tolerance, mice received peptides resuspended in PBS intravenously into the tail vein of pre-heated mice in a final volume of 200µl per mouse

at concentrations stated. As controls, mice received either PBS only i.v. or antigen plus 10μg LPS (Sigma, Poole) i.v.

2.6.4 Superantigen administration

SEB in PBS was given i.v. at a final dose of 1μg/mouse in 200μl over three consecutive days (unless stated otherwise). Syringes were disposed into incineration bins clearly marked for the disposal of SEB. Mice were maintained in individually vented cages (IVC) for the duration of experiments and all administrations carried out under ventilated hoods. At the end of experiments bedding was incinerated and cages autoclaved. Mice were sacrificed for analysis 24h after the final dose.

2.6.5 BrdU administration

Mice were treated with 2mg BrdU i.p. in a final volume of 200μl per mouse at the time-points described. Mice treated with BrdU were house in individually vented cages (IVC) and all administrations carried out under ventilated hoods. At the end of experiments, bedding was incinerated and cages autoclaved.

2.6.6 T cell transfers

Purified whole splenocytes, sorted CD4+ or CD4+CD25+CD62Lhi cells resuspended in PBS were transferred at stated numbers in a final volume of 200μl i.v. into the tail vein of pre-heated mice.

2.7 In vitro assays

2.7.1 ^{3}H-Thymidine incorporation assays

Single cell suspensions in RPMI-5 medium (number and source indicated in figure legends) were cultured in flat-bottomed 96-well microtiter plates (Becton Dickinson, CA) with the indicated antigen at indicated dose(s). After 48h culture 0.5μCi/well of tritiated thymidine (^{3}H-dThd, Amersham) was added in a volume of 25μl. 18h after addition, dThd incorporation was measured using a liquid scintillation β-counter

(Wallac). Results are expressed as a mean counts per minute (cpm) of duplicate or triplicate cultures, as indicated in the figure legend.

2.7.2 Supernatant cytokine quantification by ELISA

Single cell suspensions were cultured in 96-well microtiter plates (Becton Dickinson, CA) with indicated antigen at indicated dose(s). After 48h of culture supernatants were removed (~150µl) and used directly for ELISA or frozen @ -20^{o}C until required.

96 well MaxiSorp microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4^{o}C with 50µl/well of the relevant cytokine-capture antibody; anti-IL-2 (JES6-1A12), anti-IL-10 (JESS-2A5), anti-IFNγ (R4-6A2), and anti-IL-17 (TC11-18H10) (all BD Pharmingen) diluted to 2µg/ml in bicarbonate buffer (0.05M, pH 9.6). Plates were washed twice with PBS/0.1% Tween (Sigma) then blocked for 1h @ 37oC with 200µl PBS/1%BSA (Sigma) per well. After two washes in PBS/Tween, followed by two washes in PBS, cytokine standards were diluted in PBS/BSA were added at 100µl/well and two fold dilutions performed to give a standard curve for each plate (top and bottom concentrations: IL-2 1000-4pg/ml, IL-10 and IFNγ 100-0.4ng/ml, IL-17 10-0.04ng/ml). Samples were then added at 50µl/well in duplicate and incubated at room temperature for 2 hours. Plates were then washed with PBS/Tween before adding appropriate secondary biotinylated antibodies diluted in PBS/BSA, 100µl/well - anti-IL-2 (E56-5H4) at a final concentration of 5ng/ml, anti-IL-10 (SXC-1), anti-IFNγ (XMG1.2) and anti-IL-17 (TC11-8H4.1) at a final concentration of 100ng/ml. Plates were then incubated for 1 hour at room temperature. Once washed, ExtrAvidin peroxidase (Sigma) diluted to 2µg/ml final concentration in PBS/BSA was added (100µl/well) for 30 minutes at room temperature. After 6 washes with PBS/Tween, cytokines were detected using 100µl phosphate-citrate buffer, (prepared by adding 100µl of 10mg/ml Tetramethylbenzidine (TMB; Sigma) in DMSO to 9.9ml of phosphate-citrate buffer pH5 and 3µl of hydrogen peroxide (Sigma)). Reactions were stopped using 100µl of 2M sulphuric acid. Absorbance values were read at 450nm using a Multiskan plate reader (Labsystems, Basingstoke, UK). All cytokines were quantified with

standard curves obtained with known concentrations of recombinant mouse cytokines. Values of 0 represent undetectable levels of cytokine in culture. All antibodies and mouse standard cytokines were obtained from BD Pharmingen.

2.7.3 Intracellular cytokine staining

Cells were cultured in 96 well round bottomed plates (Costar) at stated concentrations with stated concentrations of antigen overnight. Brefeldin A (eBioscience) was added to wells at a final dilution of 1:1000 in a volume of 25µl/well. Cells were incubated for 4 hours @ 37^{o}C. Cells were then washed in FACS buffer and surface stained as described. Cells were then incubated overnight in eBioscience Foxp3 Fix/Perm buffer (200µl/well). After ~18h, cells were then stained for intracellular cytokines along with Foxp3. Cells were washed and re-suspended in 500µl FACS buffer for analysis.

2.7.4 Foxp3/BrdU staining

Cells were surface stained as described above then incubated in fixation/permeabilisation buffer (eBioscience Foxp3 kit, at a 3:1 diluent:concentrate ratio) for 18h overnight at 4^{o}C. Cells were then washed in Permeabilisation buffer (eBiosience kit), diluted to 1X in PBS. DNAse@10mg/ml (Sigma) was then diluted 1:20 with 1X Permeabilisation buffer, 200µl/sample and then incubated at 37oC for 1hour. Cells were washed in Permeabilisation buffer x 2 then stained for antibody; anti-BrdU-FITC (BD bioscience), anti-Foxp3-APC (eBioscience) both at 1:50 dilution in Perm. buffer for 20 minutes at room temperature. Cells were washed in FACS buffer then resuspended in 500µl FACS buffer for collection at a rate of ~500 events/second.

2.7.5 In vitro BrdU assays

2.7.5.1 C57BL/6 WT and MOGKO BrdU assays:

Draining lymph nodes (inguinal and paraortic) were removed from mice that had been immunized 10 days earlier with 200µg pMOG/CFA. Single cell suspensions were prepared as described previously and resuspended at 5-6x10^{6} cells/ml in RPMI-5. 500µl of cells were added per well of a 24 well plate. Antigens were added resuspended in

500µl of RPMI-5; pMOG (final concentration 10µg/ml), pOVA (10µg/ml) or anti-CD3 (1ug/ml). Cells were incubated at 37^{o}C for 24, 48 or 72h as indicated in the figure legends. For the final 18 hours of each culture (unless otherwise stated), 25µl BrdU was added to give a final concentration of 30µg/ml, diluted in RPMI-5. After the final 18h of culture with BrdU, cells were harvested from the plates into 15ml Falcon tubes and washed twice in RPMI-5 before staining for FACS analysis.

2.7.5.2 CNS-derived Treg BrdU assays:

Brain and spinal cord were removed from mice that 17 days earlier had been induced to develop EAE by immunization with 200µg pMOG emulsified in CFA. CD4+CD25+ cells were sorted as described and cultured at 3x10^{5} cells/ml, 100µl per well of 96 well round bottom plates. Irradiated splenocytes from Thy1.1+ C57BL/6 mice were used as APC and added to wells (50µl/well) at a concentration of 5x106 APC/ml. Antigens were resuspended in RPMI-5 to give the final concentrations as indicated in the figure legends by adding 50µl per well. Cells were incubated at 37^{o}C for 24, 48 or 72h as indicated in the figure legends. For the final 18 hours of each culture (unless otherwise stated), 25µl BrdU was added at a final concentration of 30µg/ml, diluted in RPMI-5. After the final 18h of culture with BrdU, cells were harvested from the plates into 5ml FACS tubes (BD Falcon) and washed twice in RPMI-5 before staining for FACS analysis.

2.7.6 In vitro suppression assays

2.7.6.1 CNS-derived Treg suppression assay:

CD4+CD25+ cells were sorted from the CNS of mice at day 17 of EAE induced with pMOG/CFA and pertussis toxin. CD4+CD25- cells were also sorted from CNS of mice at day 17 of EAE, from naïve 2D2 or naïve OT-II mice. CD4+CD25+ cells were resuspended in RPMI-5 at 2x10^{5} cells/ml and 200µl was added to the top row of a 96 well round bottom plate. 100µl of CD4+CD25+ cells were double diluted into 100µl of RPMI-5 down the plate to give two fold dilutions of the CD4+CD25+ T cells. 2x10^{4} CD25- cells in RPMI-5 were added to the wells containing Tregs, 50µl/well. Irradiated

splenocytes from naïve C57BL/6 were used as APC at $5x10^6$/ml. Antigens (pOVA or pMOG at final concentration of 10μg/ml) were diluted in APC and 50μl added to the appropriate well. Cells were cultured at 37°C for 48 hours prior to the addition of ^{3}H-thymidine. After a further 15-18 hour culture at 37°C, well were assessed for ^{3}H-thymidine incorporation as described.

2.7.6.2 Vβ5 suppression assays:

CD4+Vβ5+, CD4+Vβ5-, CD4+CD25+ (supressor) and CD4+CD25- (responder) cells were sorted by CD4+ MACS purification and FACSAria sorting to >95% purity as described. Suppressor cells were reuspended at $4x10^5$cells/ml in RPMI-5, 200μl of cells added to the top wells of a 96 well round bottom plate and double diluted into 100μl RPMI-5 down the plate. 50μl of CD4+CD25- responder cells (at $8x10^5$/ml in RPMI-5) were then added to the wells. Irradiated splenocytes from naïve C57BL/6 mice were used as APC, resuspended at $5x10^6$/ml in RPMI-5. Anti-CD3 antibody (eBioscience) was added to APC at a concentration of 4μg/ml to give a final concentration of 1μg/ml in 50μl of APC +/- anti-CD3 that was added per well. Cells were cultured at 37°C for 48 hours prior to the addition of ^{3}H-thymidine. After a further 15-18 hour culture at 37°C, well were assessed for ^{3}H-thymidine incorporation as described.

2.7.7 SEB in vitro assays

2.7.7.1 Naïve cell in vitro stimulations:

Naïve splenocytes were cultured at $5x10^6$ per well in 24-well tissue culture plates (Becton Dickinson, Oxford, UK) with either medium alone, 2.5μg/ml con A (SIGMA) or the indicated dose of SEB (SIGMA) for 96h. Cells were then analysed for expression of foxp3, using anti-foxp3 staining kits as described above. In some experiments, BrdU was added at a final concentration of 10μM for the final 12 hours and incorporation was detected by FACS as described previously.

2.7.7.2 Co-culture suppression assays:

$5x10^5$ naïve Tg4 Thy1.1+ CD4+ splenocytes were cultured alone or together with $5x10^5$ CD4-purified splenocytes from mice treated with PBS or 1ug SEB in vivo for three days prior to sacrifice. $2x10^6$ APC (B10.PL irradiated splenocytes) +/- 10μg SEB or 10μg MBP(Ac1-9) were used to stimulate cultures for 72h. BrdU was added at a final concentration of 10μM for the final 12-15 hours of culture and incorporation by Foxp3+ and Foxp3- cells was detected by FACS.

2.8 T cell hybridomas

2.8.1 Cell isolation and expansion

CD4+CD25+, CD25+CD25- and CD4+CD25+CD62Lhi cells were sorted by MACS purification and FACSAria sorting as described above. Cells were expanded in vitro using anti-CD3/anti-CD28 coated Dynabeads (Miltenyi Biotech) at a 1:1 ratio of cells:beads with the addition of 1000U/ml IL-2. After 7 days cells were washed extensively in serum free RPMI (that had been warmed to 37^oC) and counted. Cells were resuspended in serum free RPMI (SFM) at $1x10^6$ cells/ml.

2.8.2 BW5147 cell preparation

BW5147α-β- T cells (White, J 1989 JI) were grown in RPMI-5 for 4-5 days until at sufficient cell numbers. Cells were washed and counted on the day of fusion in serum-free RPMI. Cells were then resuspended in 50ml Falcon tubes (BD Biosciences) at $1x10^6$ cells/ml in warmed serum free RPMI.

2.8.3 PEG Fusion

PEG 1500 solution (Roche Diagnostics, Germany) was warmed by transferring the solution to a 15ml Falcon tube and resting in a 37^oC water bath. Expanded T cells and BW5147 cells were mixed at a 1:1 ratio – T cells were centrifuged at 1500 RPM for 5 minutes in a 50ml Falcon tube. Cells were then resuspended by the addition of an equal

number of BW5147 in SFM. Tubes were filled with SFM and re-centrifuged. The cell pellet was aspirated to dryness carefully, using a 10ml pipette (Sterilin) and gently resuspended. Using a 1ml pipette (Sterilin) 1ml of PEG 1500 was added to the cell pellet slowly (over 1 minute) while gently stirring the pellet with the pipette tip. This was repeated using 2mls of warmed SFM added slowly, over 2 minutes and again using 7mls of warmed SFM over 2 minutes. Cells were centrifuged at 1000 RPM with no brake for 5 minutes. Supernantant was aspirated as described before.

2.8.4 HAT preparation

Hypoxanthine Aminopterin Thymidine (HAT) medium (Sigma) was stored at -20^{o}C in powder form. 10ml RPMI-5% FCS was added to create a 50X solution. A 2X solution was prepared for addition to cell cultures.

2.8.5 Clonal growth and maintenance of hybridomas

Cells were counted and resuspended at 2x10^{5}/ml of the original T cell number. 100µl of cells were added per well of a 96 well flat bottom plate (Corning Incoporated). After 24h of culture at 37^{o}C 100µl of 2x HAT solution was added per well and plates returned to 37^{o}C incubators. After 6-10 days, wells were assessed for clonal growth of hybridomas, assessed as <20% of wells per plate having cells that expanded. Successful hybridomas were transferred to cell culture flasks in RPMI-5% FCS and maintained at 37^{o}C until at sufficient cell numbers to be tested for TCR-reactivity.

2.8.6 Assessing hybridoma TCR-reactivity

50µl of hybridoma cell cultures were transferred to 96 well plates (Costar) containing 100µl PL-8 B cell lines at 5x10^{5}/ml in RPMI-5% FCS +/- MBP(Ac1-9) at a final concentration of 4µg/ml. After 24h culture at 37^{o}C, 100µl of the supernatant per well was removed for detection of IL-2 by ELISA (see previous section). Positive results were indicated by production of IL-2 only in response to both APC and antigen, not to either APC or antigen alone.

2.9 Statistics

Statistical analysis performed on in vitro data used Student's t test. P values of less than 0.05 were regarded as significant. Statistical analysis on EAE mean clinical disease curves used Mann Whitney or statistical analysis based on the area under the curve.

Table 2.1: Flow cytometry antibody clones and dilutions

Antibody	Conjugate	Clone	Dilution
CD4	-FITC, -PE, -APC	L3T4	1:500
	-PercP	RM4-5	1:500
CD25	-FITC	7D4	1:200
	-PE, -APC	PC61	1:200
CD62L	-FITC, -PE	MEL-14	1:200
Foxp3	-FITC, -PE, -APC	FJK-16s	1:50
BrdU	-FITC, -APC	PRB-1	1:50
CD103	-FITC	M290	1:200
Vβ5	-FITC	MR9-4	1:200
Vβ3	-FITC	KJ25	1:200
Vβ8	-FITC	MR5-2	1:200
Vβ11	-FITC	RR3-15	1:200
Vα3.2	-PE	RR3-16	1:200
CD45.1	-FITC, -PE, -bio	A20	1:200
CD44	-PE	IM7	1:200
TCRβ	-APC	H57-597	1:200
CTLA-4	-PE	UC10-4F10-11	1:100
OX40-L	-bio	RM134L	1:200
GITR	-PE	DTA-1	1:200

3 Investigating the antigenic-reactivity of Foxp3+ Tregs in EAE

3.1 Introduction

Defective regulatory T cell function has been demonstrated in patients with MS (Viglietta et al., 2004). The pivotal role of regulatory T cells in recovery from disease has been shown in EAE by the depletion of CD25+ cells in both pMOG (McGeachy et al., 2005) and PLP models (Reddy et al., 2004; Zhang et al., 2004). Assessment of Tregs directly in the CNS has shown that these cells accumulate as disease progresses and importantly that the Tregs proliferate extensively in situ (O'Connor et al., 2007). The accumulation of Tregs in the CNS is associated with recovery from EAE and their ability to suppress auto-aggressive T cells. Tregs have been shown to initially require stimulation through the TCR by cognate antigen to become activated and suppress in vitro, although they may later suppress T cell responses in the absence of cognate antigen via bystander suppression (Thornton and Shevach, 2000). As Tregs are proliferating extensively in the CNS, it is likely that they are receiving stimulation via the TCR. While Tregs are largely believed to be self-reactive, the antigenic targets of the Tregs which accumulate in the CNS are unknown. Therapeutic targeting of Tregs is a major aim for many diseases associated with excess inflammation. Polyclonal CD4+CD25+ Tregs have been shown to suppress EAE (Kohm et al., 2002) and polyclonal expansion of human Tregs in vitro can generate functional Tregs (Earle et al., 2005). However, there is clear evidence that antigen-specific Tregs are more effective than polyclonal Tregs in suppressing organ-specific autoimmunity (McGeachy et al., 2005; Tang et al., 2004b). Determining the antigenic reactivity of Tregs that are relevant to EAE/MS is therefore essential to specifically target these cells by therapeutic strategies. This chapter describes experiments designed to determine the antigenic reactivity and suppressive capacity of the regulatory T cells which arise during EAE

3.2 Results

3.2.1 Generating regulatory T cell hybridomas

Hybridoma technology initiated with the finding that fusion of B cells to immortal cancer cells allowed the continuous generation of monoclonal antibodies (Kohler and Milstein, 1975), a discovery which has enhanced immune research dramatically. In the late 70's, Goldsby et al. (Goldsby et al., 1977) expanded the potential of hybridoma technology into the realms of T cell biology, using T cell meyloma cells. The generation of T cell hybridomas have allowed extensive investigation into T cell biology and remains an important tool in the understanding of the TCR diversity, reactivity and function (De Silva et al., 2000; Donermeyer et al., 2006; Ezquerra et al., 1990). One of the major obstacles in determining the antigenic-reactivity of Tregs, particularly from the CNS, is the low recovery of cells to test against potential peptides. The isolation of sufficient Treg cell numbers on which to perform functional assays requires a large cohort of mice which have to undergo the process of EAE. In order to overcome this we hypothesised that generation of hybridomas from CNS-derived Tregs could provide us with a pool of immortal cells expressing Treg-derived TCRs on which to assess their reactivity.

The generation of Treg hybridomas was attempted at length using a number of different TCR transgenic mouse models (as a proof of concept that the Treg hybridomas will maintain the TCR of the fused T cell – for an outline see Appendix Ia) and later with sorted Foxp3+ populations sorted from Foxp3-GFP mice. While the transgenic Treg hybrids fused successfully, Foxp3-expression was not evident in any of the hybridomas tested (Appendix Ib and Ic). Our aim was to determine the antigenic-reactivity of CNS-derived Tregs after pMOG-immunisation; therefore Tregs were sorted from Foxp3-GFP

mice (Fontenot et al., 2005b) that were in the recovery stages of EAE. None of the CNS-derived hybridomas were grown out successfully.

Our attempts to generate hybridomas which express Foxp3 were unsuccessful, however a recent study has also reported that hybrids generated from Treg populations lack Foxp3-expression (Pacholczyk et al., 2007). This group used mice which expressed a fixed TCRβ chain and a selection of TCR α-chains from a mini locus to track Foxp3+ cells based on their TCR after they had lost Foxp3-expression. Thus, while we could not use Treg hybrids to determine antigenic reactivity of CNS-derived Tregs, advances in technology may make this possible in the future.

3.2.2 Determining the antigen-reactivity of Tregs after pMOG-immunisation

As the use of hybridoma technology to generate immortal Treg clones was unsuccessful, we attempted to determine antigenic-reactivity in other ways, by using Tregs isolated directly ex vivo. The development of Tregs is believed to be dependent on self-peptide interaction in the thymus (Fontenot et al., 2005b; Jordan et al., 2001) and that Treg expansion depends on high avidity TCR ligands (Yu et al., 2008). This dogma proposes that most Tregs are selected on their bias towards higher levels of self-reactivity and that Tregs in the periphery will respond to these self antigens and thereby contribute to self tolerance against these antigens. It is not clear if immunisation with self-peptide can expand naturally arising, self-reactive Tregs and/or induce antigen-reactive Tregs in the periphery. We therefore attempted to determine the antigenic reactivity of Tregs in C57BL/6 mice after immunisation with pMOG. By utilising mice which lack the expression of the CNS protein MOG (Delarasse et al., 2003) we have also addressed the role of endogenous antigen expression on the peripheral Treg repertoire. As Tregs are believed to require self-peptide interactions in the thymus it might be predicted that

MOG-KO mice would lack MOG-reactive Tregs in the periphery and therefore MOG-reactive Tregs would not be detectable upon immunisation with pMOG.

3.2.2.1 BrdU incorporation as a measure of the antigenic reactivity of Tregs

BrdU can be used to detect cells which are undergoing DNA synthesis by incorporating this thymidine analogue into the newly formed strands of DNA. We initially confirmed that BrdU is a measure of cell division in order to utilise this method to measure antigen-induced proliferation (Appendix II). Double staining of Foxp3 and BrdU had not yet been documented, therefore a protocol was developed which allowed the detection of BrdU and Foxp3 in the same cells by combining the buffers required for Foxp3 staining and the BrdU staining protocols (see materials and methods section for details).

To determine if pMOG-immunisation could expand MOG-reactive Tregs, C57BL/6 mice were immunised with pMOG/CFA. Draining lymph nodes (DLN) were removed after 10 days and cultured in vitro with pMOG or recombinant MOG protein (rMOG) for 72h, with BrdU added for the last 18h of culture. Cells were then stained for Foxp3 and BrdU to assess the proliferative response of both Foxp3+ and Foxp3- cells. The Foxp3+ cells from pMOG-immunised mice showed increased BrdU incorporation in response to pMOG and rMOG compared to un-stimulated cultures (Fig. 3.1A). Foxp3- cells also showed increased proliferation in pMOG and rMOG stimulated cultures compared to medium alone (Fig. 3.1B). While these results suggested that pMOG-reactive Tregs are generated in response to pMOG immunisation, the levels of BrdU incorporation were very high, with >50% of Foxp3+ cells in culture responding to pMOG. The frequency of pMOG-reactive splenocytes after pMOG immunisation has been demonstrated to be in the region of 2-3% using pMOG-specific tetramers (Korn et al., 2007). It is therefore unlikely that the 50% BrdU incorporation observed in the Foxp3+ population is due to the sole proliferation of this small compartment of antigen-

reactive cells in the culture and suggests that not all of the proliferation in these cultures was antigen-induced.

3.2.2.2 Refining the BrdU staining protocol to measure antigenic-reactivity

To determine if BrdU incorporation could more sensitively reflect antigen-induced proliferation we conducted time-course experiments to assess the levels of BrdU incorporation over time in response to antigen. BrdU incorporation was detected after the addition of BrdU for the last 12 hours of 24h, 48h and 72h antigen or anti-CD3 stimulated cultures.

At the first time-point, very little BrdU incorporation was visible in any cultures (Data not shown). Foxp3+ and Foxp3- cell division was evident at 48h in culture with pMOG and anti-CD3, with ~12% of Foxp3+ cells proliferating to pMOG and 63% to anti-CD3 (Figure 3.2A, top panels). At 72h, proliferation also occurred in both Foxp3+ and Foxp3- populations in response to pMOG and anti-CD3, but to a much higher level than at 48h, with up to 55% of Foxp3+ cells proliferating in response to pMOG at this point (Fig. 3.2A, bottom panels).

Upon immunisation, antigen-reactive T cells may express immunodominant TCRs bearing particular Vβ-Dβ-Jβ rearrangments. If these rearrangements are found to be common in all mice of the same strain then they are termed public rearrangements. In C57BL/6 mice, actively induced EAE using pMOG shows preferential use of the Vβ8.2 gene segment (Fazilleau et al., 2006). In this situation, the Vβ8.2 gene segment is expressed by the ‘public’ MOG-reactive repertoire as the CD4+ T cells in most mice will show preferential use of the Vβ8 gene segment upon immunisation with pMOG. Analysis of BrdU incorporation within the Vβ8+ and Vβ8- populations of the Foxp3+ compartment demonstrated that at the 48h time-point, only the Foxp3+Vβ8+ cells had proliferated in response to pMOG (Fig. 3.2B). However, at 72h both Foxp3+Vβ8+ and

Foxp3+Vβ8- populations showed levels of BrdU incorporation comparable to whole CD4+ cells in response to anti-CD3 (Fig. 3.2B). The Foxp3-Vβ8+ and Foxp3-Vβ8- populations demonstrated similar differences in the kinetics of BrdU incorporation also (Fig. 3.2C). These results suggest that proliferation of Foxp3+ and Foxp3- cells at the 48h timepoint is reflective of expansion of Vβ8+ cells only, arguing that Foxp3+ proliferation in these cultures is antigen-specific. If the proliferation of Foxp3+ cells was induced in response to the proliferation of Foxp3- cells in the same cultures then the proliferation would not be restricted to the Vβ8+ population.

3.2.2.3 Assessing the cytokine levels in pMOG stimulated cultures

IL-2 has been shown to be required for Treg proliferation in vitro (de la Rosa et al., 2004; Thornton et al., 2004). It has more recently been reported that in vivo administration of IL-2 selectively expands Foxp3+ Tregs (Brandenburg et al., 2008). We therefore reasoned that high levels of IL-2 in culture may account for the enhanced proliferation observed by the Tregs (Fig. 3.1A). To assess the levels of IL-2 in culture we measured the IL-2 concentrations in the supernatants of LN cultures by cytokine ELISA (Fig. 3.3A). The level of IL-2 was highest after 24h stimulation with anti-CD3, although over time these levels rapidly declined presumably as IL-2 was consumed during proliferation. Stimulation with pMOG induced a small level of IL-2 after 24h and this was gradually reduced over time. The level of IL-2 in medium only cultures increased over time to levels higher than those seen in pMOG stimulated cultures. However, enhanced proliferation of Tregs was not seen in medium only cultures at any point (Fig 3.2A), arguing against IL-2-induced proliferation. The role of IL-2 in these cultures could be more readily addressed with the use of blocking anti-IL-2 antibody.

We also measured the levels of IFNγ in supernatants at each time-point, over serial dilutions of the supernatant (Fig. 3.3B). pMOG stimulated cultures showed increased IFNγ production over time, suggesting that the cells have become activated in response

to pMOG stimulation, as is the case with anti-CD3. A small level of IFNγ was detectable in medium only cultures at 24h but this was reduced over time.

From the data above it was decided that the detection of antigen-specific activation and proliferation via BrdU incorporation was optimal after antigenic stimulation for 48h in vitro.

3.2.3 The effect of endogenous MOG expression on the peripheral Treg repertoire

3.2.3.1 The antigen-reactivity of Tregs in WT vs MOGKO mice

To understand the role of self-antigen expression on the repertoire of self-antigen-reactive Tregs we compared the pMOG-reactive Treg repertoire of WT mice with MOGKO mice. Both WT and MOGKO mice were immunised with pMOG/CFA. After 10 days, cells were isolated from the DLN and stimulated with pMOG in vitro for 48h, with the addition of BrdU for the final 12h of culture. The proportion of Foxp3+ cells from MOGKO mice was repeatedly higher than the proportion of Foxp3+ cells from WT mice in both un-stimulated and pMOG stimulated cultures, although this was not significantly different (Fig. 3.4A). When stimulated with pMOG, both WT and KO Foxp3+ cells showed a significant increase in BrdU incorporation compared to their un-stimulated counterparts (Fig. 3.4B). Interestingly, at this time-point MOGKO Tregs also showed significantly enhanced BrdU incorporation compared to WT Tregs (Fig 3.4B), suggesting that Tregs generated in MOGKO mice after pMOG immunisation are more responsive to pMOG stimulation in vitro.

To determine if Tregs from MOGKO mice show differences compared to WT Tregs specifically in their response to pMOG, the above experiment was repeated with an extra group immunised with pOVA. In accordance with previous experiments, both WT and

KO Tregs from pMOG immunised mice incorporated BrdU in response to pMOG, but not in un-stimulated or pOVA stimulated cultures (Fig. 3.5A). Apart from the un-responsiveness of one culture in the MOGKO group, the trend for higher BrdU incorporation in KO vs WT Tregs was maintained. When mice had been immunised with pOVA, BrdU incorporation was only evident in pOVA stimulated cultures and no significant difference was observed between WT and KO Treg proliferation (Fig. 3.5B). These trends in Foxp3+ populations were also confirmed by analysis of cell numbers (Fig. 3.5C and 3.5D). These data again suggested that a proportion of WT and KO Tregs can respond to pMOG after pMOG immunisation and that MOGKO Tregs may show an enhanced proliferation compared to WT Tregs.

In each experiment analysed, Foxp3+ proliferation was accompanied by an increase in Foxp3- proliferation (Fig. 3.6). In this setting it would be possible that the proliferation observed in the Foxp3+ compartment could be in response to Foxp3- cell proliferation and release of IL-2. To assess if this was the case, we sorted CD4+CD25+ cells from the DLN of WT and MOGKO mice which had been immunised with pMOG 10 days earlier. These 'Tregs' (~85% Foxp3+) were then stimulated in vitro with peptide or anti-CD3 using congenically marked (Thy1.1+) irradiated splenocytes as APC. Using FACS analysis, Tregs could be identified from APC based on their lack of Thy1.1 expression. At 48h the level of BrdU incorporation in both WT and KO Tregs was significantly enhanced compared to un-stimulated controls (Fig. 3.7A). No difference in WT vs KO Treg proliferation to pMOG was observed. At 72h both WT and KO Tregs continued to proliferate in response to pMOG stimulation. However, at this later time-point WT Tregs showed a significant increase in the proportion of BrdU+ cells to a level which was significantly higher then that of KO Tregs in pMOG stimulated cultures (Fig. 3.7B). Although there was a small Foxp3- fraction within the CD25+ sorted cells, these cells did not show the same levels or trends in BrdU incorporation as the Foxp3+ cells (Fig 3.7C and D). Thus the proliferation of Foxp3+ cells in culture was unlikely to be due to the proliferation of Foxp3- cells also in culture.

These data suggested that the enhanced BrdU incorporation observed in MOGKO Tregs from unsorted T cell populations (Fig 3.4B) was the effect of a factor which has been excluded in the sorting of CD25+ cells away from other cells in the DLN. This may include the preferential production of IL-2 from another cell population in the MOGKO vs WT cultures in response to pMOG stimulation.

3.2.3.2 Treg co-cultures: Does the presence of effector T cells affect the ability of Tregs to proliferate in vitro?

To assess the contribution of effector T cell responses to driving Treg proliferation we co-cultured the sorted CD25+ from the DLN with the CD25- Teff cells also from the DLN at a 1:3 ratio (CD25+:CD25-). At 48h, Foxp3+ cells from un-stimulated cultures proliferated to levels observed in the sorted un-stimulated cultures (Fig. 3.8A). Stimulation with pMOG showed a small, but detectable increase in Foxp3+ cell BrdU incorporation (Fig. 3.8A). At 72h, the Tregs in co-culture with no stimulation had almost no detectable levels of BrdU incorporation in both WT and KO cells (Fig. 3.8B). However, both WT and KO Tregs showed high levels of BrdU incorporation to pMOG stimulation at this later time-point (Fig. 3.8B). KO Tregs now showed BrdU incorporation to the same levels as WT Tregs, suggesting that a factor which was lacking in the CD25+ sorted KO cell cultures is now available to drive the KO Tregs proliferation to a high level. Unfortunately supernatants were not available from this experiment to assess the levels of cytokines such as IL-2 in each culture; however it is difficult to see why CD25+ fraction from WT mice should contain a factor that is lacking in the KO Treg cultures.

The level of proliferation in the Foxp3- cells in the co-culture were also assessed (Fig. 3.8C and D). Similar to the Foxp3+ fraction, the Foxp3- cells in co-culture proliferated in response to pMOG in vitro. No significant difference in proliferation was observed between the WT and KO groups. It may be suggested that T cells from MOGKO mice

should show enhanced responses to pMOG compared to WT as theses cells will not have been tolerised to the antigen in vivo. However these data would suggest that there is no significant increase, or decrease, in the response of MOGKO mice to pMOG immunisation and restimulation compared to WT mice.

One study, using the retinal antigen IRBP, has suggested that that immunisation with peptide in CFA does not expand IRBP-reactive Tregs in IRBP KO mice (Grajewski et al., 2006). They suggest the suppression of experimental autoimmune uveitis (EAU) by the Tregs generated after immunisation of IRBPKO mice is due to the expansion of Tregs reactive against the mycobacterial components in the adjuvant. To determine wether the mycobacterial component of CFA was playing a role in the generation of MOG-reactive Tregs, we repeated the above experiment using WT and MOGKO mice immunised with pMOG in both CFA and IFA. We confirmed that MOG-reactive Tregs can be detected in WT and MOGKO mice immunised with pMOG in both CFA and IFA (Appendix III).

3.2.3.3 Assessing the effect of MOG on the repertoire of transgenic mice expressing MOG-reactive TCRs

2D2 transgenic mice possess CD4+ T cells expressing a TCR which is reactive against pMOG. Mice on a RAG-/- background lack nTregs and spontaneously develop autoimmunity (Hori et al., 2002; Lafaille et al., 1994; Olivares-Villagomez et al., 1998). RAG-sufficient transgenic mice are therefore used for most experiments. As discussed, RAG allows editing of TCR genes and the expression of non-transgenic TCRs in TCR transgenic mice. In the transgenic mouse models used in our lab the proportion of total CD4+ cells expressing the transgenic TCR+ cells is routinely greater than 90%, however the TCR usage specifically in transgenic Tregs has not been studied.

3.2.3.4 The effect of endogenous pMOG expression in pMOG-reactive transgenic mice

To investigate the effect of endogenous antigen expression on the repertoire of peripheral antigen-reactive Tregs we compared the expression levels of the MOG-reactive TCR in WT 2D2 and 2D2xMOGKO mice. The proportion of CD4+Foxp3+ cells in WT 2D2 mice is routinely in the region of 1-2%. This is reduced compared to non-transgenic mice where approximately 5-10% of CD4+ cells will express Foxp3. Foxp3+ cells represented ~4% of 2D2 MOGKO CD4+ cells, approximately twice the levels of WT mice (Figure 3.9A). We next assessed the proportions of Foxp3+ and Foxp3- cells that expressed the 2D2 transgenic TCR, were determined by Vβ11 and Vα3 expression (Fig. 3.9B). In WT 2D2 mice only 57% of CD4+Foxp3+ cells and 85% of CD4+Foxp3- cells expressed both the Vβ and Vα chains of the transgenic TCR (Fig. 3.9B, left hand panels). Some of the cells had lost expression of Vα3 only, possibly representing T cells which had undergone TCRα revision induced by recognition of self-antigen. A larger proportion of cells no longer expressed either of the transgenic TCR chains, but still expressed a TCR of unknown specificity as determined by staining with a pan TCRβ antibody (data not shown).

In comparison, the Foxp3+ and Foxp3- populations from 2D2 MOGKO mice maintain the expression of the transgenic TCR to much higher levels (Fig. 3.9B, right hand panels). WT 2D2 mice therefore have fewer Tregs and almost half of these cells did not express the MOG-reactive transgenic TCR. This can be attributed to the endogenous expression of MOG in the WT mice, as MOGKO 2D2 mice have a higher proportion of Tregs almost all of which express the pMOG-reactive TCR.

To determine if the revision of the transgenic TCR in WT 2D2 mice compared to MOGKO 2D2 mice was due to peripheral antigen encounter, the thymi of WT and MOGKO 2D2 mice were removed to assess TCR expression on thymocytes from each mouse (Fig. 3.10). Double staining for CD4 and CD8 revealed that thymocytes from WT

mice had a reduced proportion of CD4 single positive cells (CD4 SP) compared to MOGKO 2D2 thymocytes (Fig. 3.10A). However, CD4 SP cells from WT mice showed a higher proportion of Foxp3+ cells, suggesting that while WT mice had fewer CD4+ cells, more of the CD4 SP cell that remained were Foxp3+, arguing for their selective survival from negative selection or for their enhanced selection due to the presence of self antigen expression. While the proportion of Foxp3+ cells was higher in the CD4 SP fraction of WT mice, the total number of CD4+Foxp3+ cells was higher in MOGKO mice compared to WT mice as there is a higher proportion (~6 fold increase) of CD4 SP cells in KO mice vs. WT mice (Fig. 3.10A).

Expression of the transgenic TCR was determined by staining for the transgenic Vβ chain, Vβ11 and for the expression of alternative TCR Vβ chains using a pan-TCRVβ antibody (Fig. 3.10B). Foxp3+ thymocytes from both WT and MOGKO showed little evidence of TCR revision, however, Foxp3- thymocytes from WT mice showed a greater loss of transgenic TCR expression compared to MOGKO Foxp3- thymocytes (Fig. 3.10B). The levels of TCR Vβ11 expression were compared in WT vs. MOGKO mice and showed that the Foxp3+ and Foxp3- thymocytes from MOGKO mice expressed a higher level of the transgenic TCR compared to WT mice (Fig. 3.10C). Foxp3- thymocytes from WT mice showed a general shift in the intensity of Vβ11 expression compared to WT Foxp3- thymocytes. However, the majority of MOGKO Foxp3+ thymocytes expressed similar levels of the transgenic TCR, a small population expressed enhanced levels of Vβ11 (Fig. 3.10C).

These data suggest that thymic encounter with MOG reduces the proportion of CD4 SP cells that are positively selected in MOG-reactive transgenic mice. Furthermore, MOG expression initiates TCR revision in Foxp3- thymocytes, while in the periphery, both Foxp3+ and Foxp3- cells are seen to lose expression of the transgenic TCR. This was not evident in MOGKO mice and suggests that endogenous antigen expression may reduce the thymic and peripheral self-antigen reactive Foxp3+ repertoire.

3.2.3.5 Tregs that have lost the expression of the transgenic TCR do not proliferate to antigen in vitro

The response of Tregs expressing the transgenic TCR vs non-Tg TCR was assessed by sorting CD4+CD25+ lymphocytes (85% Foxp3+) from naïve 2D2 mice and stimulating them in vitro with pMOG. Of the CD25+ cells which were un-stimulated, only ~50% expressed Vβ11 in confirmation with the previous experiments (Fig. 3.11A). CD25+ cells stimulated with pMOG and anti-CD3 maintained the proportions of Vβ11+ vs Vβ11- cells at a 50:50 ratio (Fig. 3.11A).

To determine if Vβ11+ cells responded better to pMOG over Vβ11- cells BrdU was added to the cell cultures to assess proliferation. When compared to un-stimulated cultures, Vβ11+ cells showed a significant increase in BrdU incorporation in response to pMOG stimulation while Vβ11- cells did not (Fig 3.11B). Both Vβ11+ and Vβ11- populations proliferated in response to anti-CD3, demonstrating that the Vβ11- cells are expressing a functional TCR, although the levels of BrdU incorporated was reduced in Vβ11- vs Vβ11+ cells (Fig 3.11B). This demonstrates that Tregs from WT 2D2 mice which do not express Vβ11 are less responsive to pMOG.

3.2.4 Antigenic reactivity of CNS derived Tregs in active EAE.

3.2.4.1 CNS-derived Treg reactivity: 3H-Thymidine incorporation

Tregs have been shown to accumulate and proliferate in the CNS during EAE (McGeachy et al., 2005; O'Connor et al., 2007). It was not clear if the Tregs that arise/expand in the CNS in response to pMOG/CFA immunisation were also pMOG-reactive, reactive against other CNS components or indeed reactive against CFA components. As Tregs were largely believed to be anergic to in vitro stimulation it would be difficult to determine their antigenic responsiveness by proliferation

(Takahashi et al., 1998). However, it is now clear that Tregs can be induced to proliferate in vitro in response to antigenic stimulation (Tang et al., 2004b).

In order to study the reactivity of Tregs isolated from the CNS during EAE, mice were sacrificed at day 16 post EAE-induction. Lymphocyte populations were then sorted into CD4+CD25+ (Tregs) or CD4+CD25- (Teff) to high purities, routinely >90%. The CD4+CD25+ Treg populations consistently showed ~85-90% Foxp3+ cells. Due to the difficult nature of obtaining lymphocytes from the CNS, coupled with the loss of cell numbers during the sorting process, very few CNS-derived Tregs could be recovered from any one experiment. From approximately 16 mice in the recovery stage of disease the average number of CD4+CD25+ cells that could be recovered from the pooled CNS samples was 0.5-1x106 total cells. Therefore, only low cell numbers (1x104cells/well) could be used to test the in vitro antigenic-reactivity of the Tregs by thymidine incorporation.

In initial experiments, exogenous IL-2 was added to the cultures at 1000U/ml, a dose pre-determined in the lab to maintain Foxp3+ expression well in vitro. However, when cultured in the presence of IL-2 alone proliferation was observed in both the CNS-derived Treg and Teff cells and proliferation of either subset was not significantly enhanced by the addition of pMOG to the cultures (Fig. 3.12). Exogenous IL-2 induced proliferation in naïve CD4+CD25+ to a low level, but did not induce proliferation in CD4+CD25- populations. These data emphasise that in naïve T cells, Tregs are sensitive to the addition of exogenous IL-2, while both Treg and Teff isolated from a site of inflammation are highly responsive to IL-2 (Figure 3.12). In a repeat experiment the addition of exogenous IL-2 alone again induced proliferation of both CNS CD25+ and CD25- and naïve CD25+ populations (Fig. 3.13A). The proliferation of both CNS derived and naïve CD25+ populations was not significantly enhanced by addition of pMOG or rMOG, however the CNS CD25- populations did show an enhanced proliferation to pMOG (Fig. 3.13A). In cultures where no exogenous IL-2 was added to the cultures proliferation could be detected in the CNS-derived CD4+CD25+ population

in response to pMOG and to a lesser extent, rMOG (Fig. 3.13B). CNS CD4+CD25- populations proliferated in response to pMOG but also showed a good proliferative response to rMOG (Fig. 3.13B). Neither of the naïve populations proliferated in response to pMOG or rMOG.

These results suggested that in the absence of exogenous IL-2 CNS-derived CD4+CD25+ and CD25- populations show a small, but detectable level of proliferation in response to pMOG in vitro when EAE was induced with pMOG/CFA. CNS-derived CD25- populations can also proliferate in response to whole MOG protein, while CD25+ cells do not. However, while sorting for Tregs based on CD25 expression generated a population of cells of which ~85% expressed Foxp3, it is difficult to rule out that the proliferation observed in response to pMOG was due to the contaminating 15% of Foxp3- cells. We therefore required a method to determine the proliferation of the Foxp3+ cells specifically.

3.2.4.2 CNS-derived Treg reactivity: BrdU incorporation

To detect Foxp3+ cell proliferation we again utilised BrdU staining. The benefit of BrdU is that it allows detection of proliferation at a cellular level, while thymidine incorporation only measures the proliferation at a population level. As in previous experiments, mice were actively immunised for EAE induction using pMOG/CFA and Treg/Teff populations sorted from the CNS at day 16 (Fig. 3.14). Cells were then cultured with various peptides to test antigen-induced BrdU incorporation to antigens from the CNS, components of CFA and self antigens, including heat shock proteins (HSP's) (Table I). Heat shock proteins are highly conserved, with mammalian and prokaryotic HSPs showing up to 50% homology. HSPs are consitutively expressed in the CNS and up-regulated in MS plaques (Selmaj et al., 1992). HSP65 is the immunodominant mycobacterial protein which is recognised by the immune system after immunisation with CFA, while HSP60 is an endogenous HSP expressed in mice

and up-regulated response to inflammation (Birnbaum, 1995). The regulatory role of HSPs have been described in adjuvant induced arthritis (Anderton et al., 1995; Quintana et al., 2003) and in NOD mice (Quintana et al., 2002). Furthermore, phase II clinical trials have shown a beneficial effect of treatment with a peptide from HSP60 in type 1 diabetes (Raz et al., 2007). It was therefore important to determine if the Tregs from the CNS were reactive against components of myelin or against components of the adjuvant which could contribute to non-specific regulation.

Tregs or Teff were cultured in vitro for 48h with BrdU added for the final 18h of culture. As mentioned, CD4+CD25+ cells routinely contain ~85-90% Foxp3+ cells. After in vitro culture this proportion is reduced to ~70%, with a population of Foxp3- cells evident upon intracellular Foxp3 staining (Fig. 3.15A). Whether the appearance of these cells represented an outgrowth of Foxp3- cells, death of a proportion of Foxp3+ cells or loss of Foxp3-expression by cells which initially were Foxp3+, is unknown. However, by gating on the Foxp3+ population in culture (CD25+Foxp3+ refers to the population indicated by the red box in Fig. 3.15A) we can assess the proportion of Foxp3+ cells which have incorporated BrdU in each culture. As was the case in the thymidine incorporation assays, Foxp3+ cells showed the greatest BrdU incorporation to pMOG and rMOG (Fig. 3.15B), but also incorporated BrdU in response to the CFA component mycobacterium purified protein derivative PPD (Fig 3.15C). Interestingly, exposure to PPD has been previously suggested to suppress EAE via a mechanism involving CD4+ T cells (Ben-Nun et al., 1993). The data presented here also suggests that immunisation with mycobacterial components can drive Treg proliferation.

In the sorted CD25+ cultures the contaminating Foxp3- compartment also showed a degree of proliferation to the same antigens as the Foxp3+ cells (Fig. 3.15C, black bars). Thus, while Foxp3+ cells show responsiveness to some antigens from the immunising components, Foxp3- cell proliferation could also contribute to the levels of Foxp3+ cell proliferation observed in previous experiments. This raises the issue of wheter the Teff proliferation in response to the antigens results in IL-2 production, which could

indirectly induce Treg proliferation. As the Foxp3- population is such a small component of the sorted CD25+ population it would be unlikely that stimulation of this small fraction of cells would generate the levels of IL-2 to induce proliferation of 30% of the Foxp3+ cells in culture. The role of IL-2 in culture could again be assessed using anti-IL-2 blocking antibody. The data presented here would suggest that Tregs in pMOG induced EAE show a degree of proliferation to pMOG and rMOG, but also to components of CFA, which may contribute to suppression in a bystander fashion.

In vitro responses of CD4+CD25- populations derived from the CNS were also investigated (Fig. 3.16). The CD25- populations showed a high degree of spontaneous BrdU incorporation (in culture with medium alone) and this was not significantly enhanced by the addition of antigen to the cultures. This demonstrated that CD25- cells from the CNS are in a highly activated state upon isolation from the CNS (Fig. 3.16A). When supernatants from the cell cultures were assessed for cytokine production by ELISA it was evident that only CD25- cells cultured with pMOG and rMOG produced high levels of IFNγ, while low levels of IFNγ could be detected in PPD and HSP60 stimulated cultures (Fig. 3.16B). Thus, while Teff cell responses could not be distinguished at the level of proliferation, effector cytokines are released only in response to certain antigens, suggesting a level of activation induced by these antigens not seen in the other cultures. Interestingly, when the CD25+ cell cultures were assessed for IFNγ production, low levels of IFNγ (10-fold less than in CD25- cultures) were detected in the pMOG and PPD stimulated cultures, but not in the rMOG stimulated cells (data not shown). Again, these data suggest that both CD25+ and CD25- populations from the CNS of pMOG/CFA immunised mice respond to pMOG in vitro, but clearly demonstrate that proliferation is not the most sensitive measurement of antigen-induced responses, particularly in CD4+CD25- cells isolated from an inflamed site.

3.2.4.3 CNS-derived Treg suppression assays

While the Tregs from the CNS were shown to be responsive to pMOG, it was not clear if these cells could suppress MOG-reactive effector cells or if suppression could only occur after stimulation with pMOG. CNS-derived CD4+CD25+ cells were therefore assessed for suppressive effects on proliferation. Responder cells were either their CD4+CD25- counterparts or naïve CD4+CD25- cell either from pMOG-reactive 2D2 mice or pOVA-reactive OT-II TCR transgenic mice.

When cultured with 2D2 responders and pMOG, CNS-derived Tregs dramatically suppressed the proliferation of the pMOG-reactive TCR transgenic cells, even at very low Treg:responder cell ratios (Fig. 3.17A). However, when CNS Tregs were cultured with naïve OT-II responders and pOVA there was no evidence of suppression (Fig. 3.17A). To determine if Tregs derived from the CNS of pMOG/CFA immunised mice required stimulation with pMOG to exert their suppressive effect, OT-II responders were cultured with CNS-derived Tregs and both pOVA and pMOG were added to the cultures. CNS-derived Tregs did not show suppression of OT-II responders even when pMOG was added to cultures (Fig 3.17A).

The proliferative response of CNS effectors could not be suppressed by either naïve or CNS-derived Tregs (Fig. 3.17B). Indeed, proliferation appeared enhanced at high Teff:Treg ratios, suggesting the Tregs may have proliferated in these cultures. The data here demonstrate that Tregs from the inflamed CNS show a response to pMOG. This is emphasised by the fact that these Tregs can suppress pMOG-induced proliferation of pMOG-reactive Teff in vitro. However, the CNS-derived Tregs do not suppress T cells of other antigenic-reactivities.

3.2.4.4 TCR analysis of CNS-derived Tregs

Upon immunisation, antigen-reactive T cells bearing particular TCR Vβ chains will expand preferentially. This has been documented for Vβ8+ T cells in actively induced EAE using pMOG. To assess if the TCRs used by Tregs in the CNS during active EAE reflect the public expression of Vβ8 we analysed the TCR usage of each population isolated from the CNS of mice in the recovery phase of active EAE. Between 15-20% of CD4+ T cells from the CNS expressed Vβ8+ TCRs, however, this was not enhanced compared to the periphery of either the same mice or naïve mice or mice which had developed EAE (Fig. 3.18A). The proportion of Vβ8+ cells which expressed Foxp3 was also not enhanced in the CNS (Fig 3.18B). Later investigations in the laboratory determined that Vβ8+ T cells preferentially expand in both the Treg and Teff compartments over the course of the disease, although this effect was not dramatic (published, JI paper).

Interestingly the analysis of Foxp3+ cells within each of the Vβ+ compartments highlighted that Vβ5+ T cells showed a dramatic skewing towards Foxp3+ cells, with approximately 1:3 of CD4+Vβ5+ cells expressing Foxp3 (Fig 3.18B). The mechanism behind this Foxp3+ enrichment in Vβ5+ cells is explored in Chapter 5.

3.3 Discussion

While it is has been demonstrated that Tregs are important in the prevention and/or suppression of EAE (Furtado et al., 2001; McGeachy et al., 2005; Reddy et al., 2004; Zhang et al., 2006) the origin, antigenic-reactivity and dynamics of CNS-relevant Tregs were unclear. This chapter describes a number of attempts to define the antigenic-reactivity of Tregs from the CNS and DLN of mice with EAE induced using the 35-55 epitope of MOG. We have also investigated how the expression of endogenous MOG can affect the generation of self-reactive Tregs and how this might affect their capacity to respond to immunisation with MOG.

To determine the antigenic-reactivity of EAE-relevant Tregs we needed access to a large number of Tregs derived from the CNS. To overcome this, we attempted to generate Treg hybridomas. We could not identify any other studies at that time which had attempted to produce hybridomas specifically from Treg populations and unfortunately, none of our attempts resulted in successful hybridoma generation. If we had been successful in generating hybridomas from our CNS-derived Treg populations it is unlikely that we could determine if they originally were 'true' Foxp3+ cells, as expression of Foxp3 appeared to be lost in the fusion process.

A recent paper from Pacholczyk et al (Pacholczyk et al., 2007) has demonstrated that the lack of Foxp3-expression by hybridomas is one shared by other labs. They have begun to answer the question of whether the hybridomas generated from sorted Treg populations were of Treg origin by using a TCR-mini mouse. These mice express a fixed TCRβ chain and generate a number of TCRα chains selected from a cassette of TCRα genes. This allows identification of T cell origin by comparing the CDR3 sequence of the TCR used by a population with the CDR3 sequence expressed by the cell of interest. Their conclusion was that the TCRs expressed by the Treg hybrids were derived from the pool of TCRs expressed by the Foxp3+ cells in the starting population. Similar to our

expansion protocols, while the initial Treg population was highly pure for Foxp3-expression, the expanded Foxp3+ population still contained contaminating Foxp3- cells. With this finding it would be of great advantage to use this technology in our setting of EAE to confirm that the CNS-derived Treg-hybrids are derived from 'true' Foxp3+ Tregs.

The ultimate goal of these studies would be to test these Treg-hybrids for reactivity against potential peptide candidates, including CNS-derived epitopes and other self molecules, such as heat shock proteins (HSPs) to determine their reactivity. This would then allow us to expand these Tregs directly in vitro, via mechanisms that will be discussed further in chapter 4.

While the immunisation of mice with self-peptide in CFA has been shown to activate Teff which are reactive to the peptide it was unclear if peptide-reactive Tregs could also be triggered in the autoimmune inflammatory response. As we could not successfully generate Treg hybridomas, we developed a model where we could measure antigen-induced proliferation of Tregs directly ex vivo. By utilising thymidine and BrdU incorporation as a measure of detecting antigen-induced Treg proliferation we identified that a proportion of Foxp3+ cells from the DLN of mice that had been immunised with pMOG incorporated BrdU in response to 48h in vitro stimulation with pMOG (Fig 3.2A). This reflected the proportion of pMOG-specific cells detected using tetramer staining by the Kuchroo group (Korn et al., 2007).

Using the protocol we had developed we showed that after pMOG immunisation, Tregs which are reactive to pMOG in vitro can be identified in mice which lack the endogenous expression of pMOG (Delarasse et al., 2003). In un-sorted DLN cultures Tregs from MOGKO mice, compared to WT Tregs, demonstrated a small but consistent increase in the proportion of cells which proliferated in response to pMOG in vitro (Fig. 3.4). However, this capacity for enhanced responsiveness of KO Tregs to pMOG stimulation was lost when Tregs were sorted and cultured alone (Fig. 3.7A).

Furthermore, of the CD4+Foxp3+ T cells from MOG-reactive transgenic mice (2D2) only 50% expressed the transgenic TCR (Fig 3.9B). However 2D2 mice which lacked endogenous MOG expression not only had higher levels of Foxp3+ cells (Fig 3.9A), but also showed that up to 96% of the Foxp3+ cells expressed the MOG-reactive TCR (Fig. 3.9B). These data suggest that endogenous MOG-expression affected the proportion of Tregs which expressed pMOG-reactive TCRs. Importantly, it was shown that T cells which lack the transgenic TCR do not respond to pMOG stimulation in vitro (Fig. 3.10).

Pacholczyk et al. have argued that commitment to the Treg vs. Teff lineage is not determined by TCR avidity based selection in the thymus. They suggest that the two populations express the same TCRs, but these are asymmetrically distributed between them (Pacholczyk et al., 2006). The implications of the experiments described in this chapter are that thymic expression of cognate self antigen is not necessary for the generation of Foxp3+ self-antigen reactive Tregs. This would argue against the dogma that Tregs are selected in the thymus based on the high avidity of their TCRs for self antigen (Apostolou et al., 2002; Jordan et al., 2001; Yu et al., 2008) and proliferate in response to self-antigen encounter in the periphery (Hsieh et al., 2004; Walker et al., 2003).

However, Tregs that are reactive to non-self antigens clearly exist, for example CD4+CD25+ Tregs which contribute in the regulation towards parasite and mycobacterial components (Belkaid and Rouse, 2005) and during viral responses (Suvas et al., 2003). It has been proposed that while these Tregs may be able to suppress the inflammatory responses, in some cases this may be due to the ability of Tregs to respond to weakly cross-reactive antigens (Larkin et al., 2007) or the non-specific activation of Tregs via TLR stimulation from components of the immunising adjuvant (Grajewski et al., 2006). The Ignatowicz group argues that self-antigen interactions are not exclusively required for the generation of Tregs and that Tregs are selected as part of the natural CD4+ T cell repertoire (Pacholczyk et al., 2002). Furthermore, they suggest that the ligand which selects a cell to become a Treg in the thymus may differ from the ligand

which activates the same Treg in the periphery and that self-reactive Tregs are rarely found in the periphery (Pacholczyk et al., 2007).

Two studies using antigen-KO mice have concluded that endogenous antigen-expression is required for the generation of antigen-reactive Tregs. One study used a model of autoimmune gastritis, in mice lacking the major gastric antigen H/K+ ATPase (Zwar et al., 2006) and the other using mice lacking interphotoreceptor retinoid-binding protein (IRBP) in experimental autoimmune uveitis (EAU) (Grajewski et al., 2006). Both demonstrated that while the KO mice lacked antigen-specific Treg after antigen-immunisation, the mice possessed Tregs capable of suppressing disease. Interestingly, the Caspi group could not generate Tregs capable of suppressing EAU when IRBP was immunised in IFA (Grajewski et al., 2006). Their suggestion is that Tregs are activated by the mycobacterial components of CFA, either via activation of mycobacterium-reactive Tregs or polyclonal Treg stimulation via TLR ligation. While we could detect PPD-induced proliferation of CNS-derived Tregs after pMOG/CFA immunisation, we detected a much higher level of proliferation in response to pMOG (Fig 3.14). Importantly, we also detected pMOG-induced proliferation in both WT and KO Tregs after pMOG/CFA immunisation (Fig. 3.4) suggesting that the polyclonal pMOG-reactive Treg repertoire is not affected by the lack of endogenous MOG expression.

One study has reported that the Treg TCR repertoire is more diverse than that of non-Tregs (Pacholczyk et al., 2006). They argue that the higher diversity of Treg TCRs allows these cells to match the specificites of auto-reactive effector T cells. Importantly, this study determined that the Treg TCR repertoire was not adversely affected by the lack of tissue-specific antigen expression, which would corroborate with our finding that MOG-reactive Tregs can be identified in MOGKO mice.

While our findings are intriguing, our studies are limited as the detection of antigen-induced proliferation is assessed by BrdU incorporation of all cells in culture, not solely in MOG-reactive Tregs. It is particularly difficult to assess Treg proliferation in cultures

which also contain contaminating, proliferating Foxp3- populations, as is the case in most of our Treg assays. It is important to mention that sorting of 100% pure Treg populations may not be beneficial in this setting as Tregs are classically anergic to in vitro stimulation in the absence of IL-2 (de la Rosa et al., 2004; Thornton et al., 2004). It is likely that the proliferation of a small Foxp3- fraction in vivo provides the extra signals (such as IL-2) required to drive Treg proliferation. The detection of pMOG-specific cells in vitro, and in vivo, could be overcome by the use of MOG-reactive tetramers, a protocol which is currently being developed in the lab. Another important next step would be to assess the suppressive capacity of the pMOG-reactive Tregs from both WT and MOGKO mice. This could be assessed in vitro, but also in vivo by sorting the Tregs from WT and KO mice after pMOG immunisation and assessing their ability to suppress pMOG induced disease by adoptive transfer of the Tregs.

By isolating Tregs directly from the inflamed CNS we have demonstrated that active induction of EAE results in Teff (Foxp3-) and Treg (Foxp3+) populations in the CNS which have the capacity to respond to the immunising antigen (Fig. 3.14B). The cells isolated from the CNS also demonstrate a low level of responsiveness to components of the adjuvant, in this case CFA (Fig. 3.15C). Tregs from the CNS demonstrated a proliferative capacity to pMOG and suppressed MOG-reactive TCR transgenic effector T cells responses in vitro (Fig. 3.17A). However, CNS-derived Tregs could not efficiently suppress the proliferative response of CNS-derived Teff cells in response to pMOG (Fig. 3.17B).

Our studies are in agreement with those of Korn et al. (Korn et al., 2007), utilising Foxp3gfp knock-in mice and MOG(35-55)/I-Ab MHC class II tetramers to track MOG-reactive Tregs during MOG-induced EAE. They found that MOG-reactive Tregs accumulated in the CNS just before the peak of disease and that this population was significantly expanded after in vitro culture with pMOG. Similar to our studies, CNS-derived Tregs suppressed naïve 2D2 cells, but not CNS-derived effector cell proliferation to pMOG in vitro. This may be reflective of the effector cells from

inflamed sites being in a heightened activation state, therefore making suppression of their response more difficult than in naïve T cells.

It is interesting to note that in our studies the proliferation of TCR transgenic cells is much more robust compared to the proliferation of CNS-derived T cells (compare Y axis of Fig. 3.17A vs B) yet the response of CNS Teff is not affected by the presence of Tregs. It may be suggested that Tregs require a stronger proliferative response of Teff cells to become activated to suppress, however the culture of OT-II Teff with pOVA and pMOG would argue against this (Fig. 3.17A). It has been suggested that once Tregs become activated by TCR stimulation they may suppress via bystander mechanisms (Fisson et al., 2006; Grajewski et al., 2006; Thornton and Shevach, 1998) while others suggest that Tregs may only suppress Teff with the same cognate specificities (Tanchot et al., 2004). While the data from pOVA plus pMOG stimulated cultures suggest that the latter may be the case (Fig. 3.17A), this may reflect limitations of the assay, such as availability of pMOG for the Tregs to encounter in vitro. It may be possible that if CNS-derived Tregs were previously activated with pMOG before addition to the OT-II-pOVA suppression assay then they may have been able to suppress the level OT-II cell proliferation.

As Th17 cells are now suggested to be the main effector T cell population involved in the pathology of EAE it is interesting to note that some studies are reporting that effector Th17 cells cannot be as efficiently suppressed as other Th subsets (O'Connor et al., 2007; Stummvoll et al., 2008). Studies from our lab have investigated the ability to suppress effector cell cytokine production in the CNS (O'Connor et al., 2007). We found that while Tregs from the CNS effectively suppressed CNS-derived Teff IFNγ production, levels of IL-17 in the same cultures was not affected (Appendix Publication, Fig. 4). This may be due to the release of cytokines by the CNS-derived effectors, such as IL-6, which has been shown to block de novo Treg generation (Dominitzki et al., 2007).

In the study by Korn et. al. the addition of exogenous IL-6 to in vitro suppression assays using CNS-derived Tregs and 2D2 effector T cells abrogated suppression by 50% (Korn et al., 2007). However, a combination of IL-6 and TNFα completely blocked the suppressive capacity of CNS-derived Tregs. In their CNS-Treg:CNS Teff suppression assays, high levels of IL-6 and TNFα were also detected in culture. Levels of IFNγ in these cultures were not assessed; however, addition of exogenous IFNγ to the assays only demonstrated minor effects on suppression. These studies strongly suggest that the ability to be suppressed by Tregs in the CNS is different in IFNγ-producing populations compared to IL-17-producing populations, perhaps due to their sensitivity to IL-6 in the CNS-environment.

While unable to suppress CNS-derived Th17 effector cells in vitro, Tregs must eventually acquire the potential to suppress the effector T cell response in the CNS in vivo as they are ultimately responsible for the recovery phase of disease. The conditions in vivo may provide an environment where Tregs become activated and their proliferation will increase the proportion of regulatory cells relative to effector cells over time. Indeed, Korn et al. demonstrated that the ratio of pMOG-reactive Tregs:Teff is increased from 1:13 at disease peak to 1:4 during recovery (Korn et al., 2007). This was attributed to the reduced levels of IL-6 at this point. The balance of inflammation vs. regulation is obviously more complex than the Teff:Treg ratio and involves CNS-resident cells and the CNS microenvironment (Dittel, 2008).

While these studies have investigated the antigenic-reactivity of Tregs in actively induced EAE there is obviously no immunising antigen in patients with MS as disease is triggered by an unknown stimulus. Similar investigations to those described have been conducted in the lab using passive induction of EAE (O'Connor et al., 2007). This involves transfer of CD4+ T cells isolated from pMOG-immunised mice and activated in the presence of polarising cytokines in vitro. In this setting, Tregs from the CNS showed a much reduced proliferative response to pMOG in vitro (Appendix Publication, Fig. 3A), however the Tregs isolated from the CNS during passively-induced EAE could still

suppress pMOG-induced 2D2 cell proliferation (Appendix Publication, Fig. 3C). Irrespective of the mechanism of EAE induction, we have demonstrated that it is possible to detect antigen-induced responsiveness in regulatory T cells by in vitro mechanisms. This will be important in identifying the antigenic-reactivity of Tregs in patients with MS, perhaps by isolating regulatory cells from peripheral blood, as will be crucial if these cells are to be specifically targeted in therapies. It will also be crucial to understand if Tregs and Teff during the disease course are reactive against the same antigens and/or epitopes of these antigens. It would be difficult to specifically target regulatory cells via antigen-based therapies if Teff cells can respond to the same antigen. If this is the case it would be necessary to develop strategies to specifically target Tregs, perhaps through the use of antibodies which will deliver antigen to the appropriate subset of APC which can stimulate Tregs preferentially, as has recently been suggested in one study, identifying CD11c+CD11b+ DC in peptide-induced tolerance to EAE via the intravenous route (Li et al., 2008).

Ultimately, it may be that disease-relevant Tregs do not equal antigen-specific Tregs, although as will be discussed in later chapters, this may be sufficient for Treg-based therapeutic strategies. It is likely that combination therapy involving the targeting of Tregs along with cytokine blockade will be required to generate an environment in the CNS where regulation will be favoured. The central aim would be to tip the balance towards specific CNS-targeted regulation at an earlier time-point, before any overt inflammation occurs. Maintenance of this regulatory state will also be key to induce long-term tolerance to myelin antigens and to prevent relapses of disease. Understanding the antigenic reactivity and requirements for selective expansion of CNS-relevant Tregs is therefore crucial for this to be achieved.

Taken together, these data demonstrate that immunisation with peptide results in the appearance of Foxp3+ Tregs in the CNS and show in vitro responsiveness to the immunising antigen. CNS-derived Tregs can suppress naïve T cell antigen-induced proliferation in vitro, but not proliferation of effector T cells isolated from the inflamed

CNS. The presence of endogenous antigen is not required for the expansion of antigen-reactive Tregs. However, the endogenous expression of antigen may have a detrimental effect on the generation of self-antigen-reactive Tregs, although the mechanisms and level at which this effect would be mediated are unclear and require further investigation.

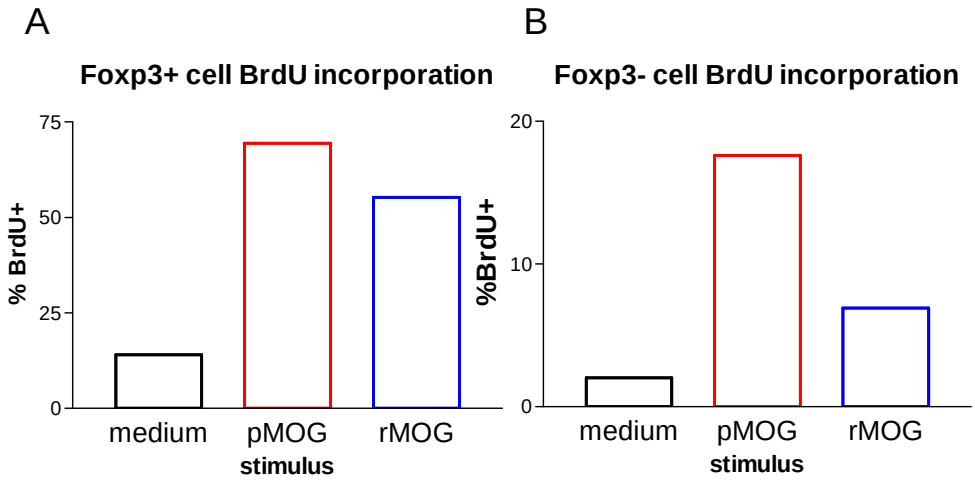


Figure 3.1: Foxp3+ and Foxp3- cells from C57BL/6 mice immunised with pMOG proliferate in response to pMOG re-stimulation in vitro. DLN were removed from WT C57BL/6 mice which had been immunised with pMOG/CFA 10 days earlier and stimulated in vitro with medium, pMOG (10µM) or rMOG for 72h. BrdU was added for the last 18h of culture. Levels of BrdU incorporation and Foxp3 expression were assessed by FACS, gated on live CD4+ cells. Bars show individual wells of pooled samples. Data is representative of three individual experiments with similar results.

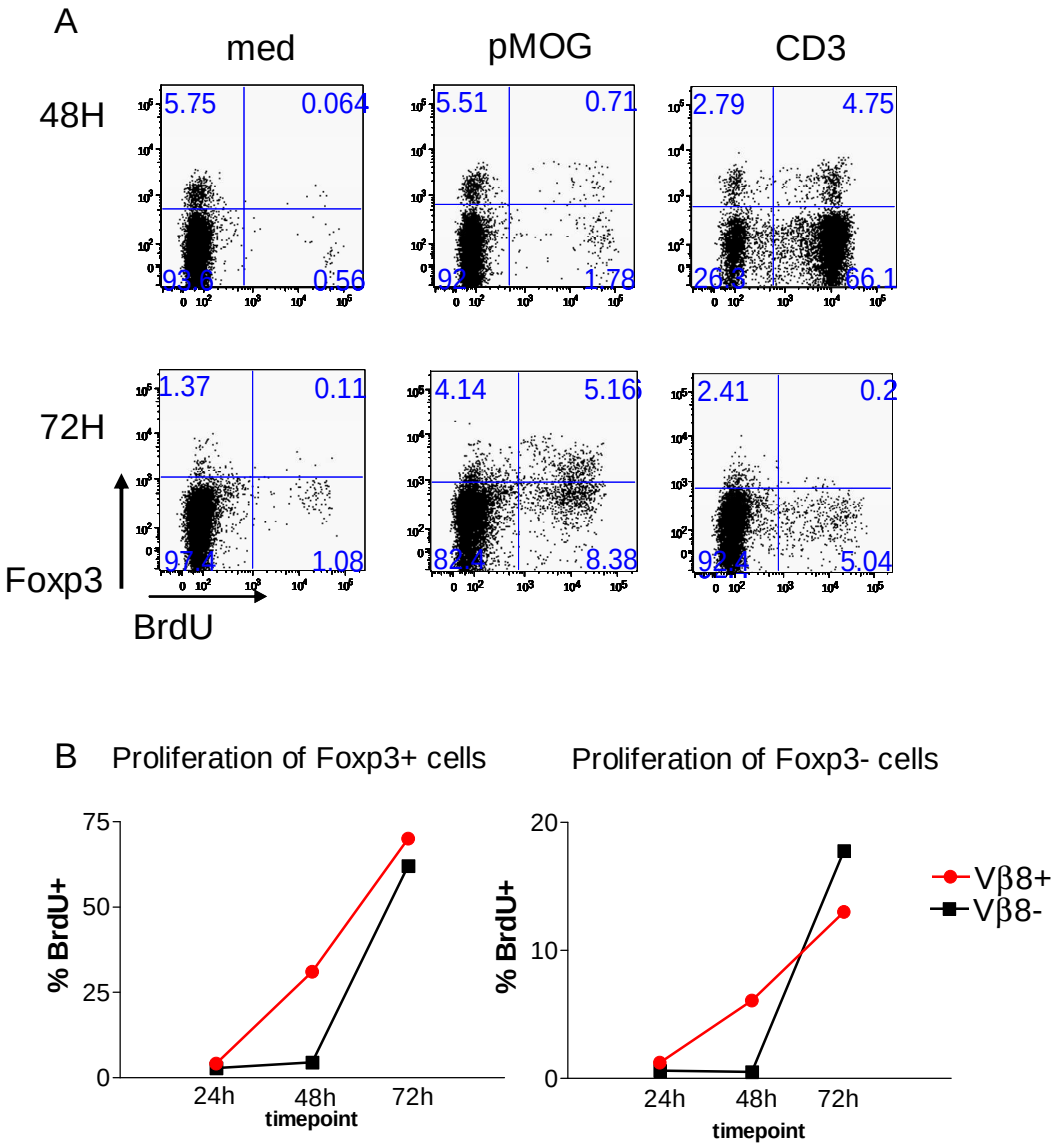


Figure 3.2: Time-course analysis of BrdU incorporation in response to in vitro stimulation with pMOG to determine antigen-induced proliferation. DLN of B6 mice which had been immunised with pMOG/CFA 10 days earlier were removed. Whole lymphocytes were cultured @ $5x10^6$/ml, 1ml per well of 24 well plate. Cells were left un-stimulated or stimulated with pMOG (10μM) or αCD3 (1μg/ml). BrdU was added at 12, 24 and 48h to assess BrdU incorporation over the next 12 hours. A: 48H and 72H time-point FACS plots of BrdU incorporation in Foxp3+ and Foxp3- cells, gated on live CD4+ lymphocytes. B: %BrdU incorporation of CD4+Foxp3+Vβ8+ cells (red line) and CD4+Foxp3+Vβ8- cells (black line) in Foxp3+ and Foxp3- gated populations. Data representative of three independent experiments showing similar results.

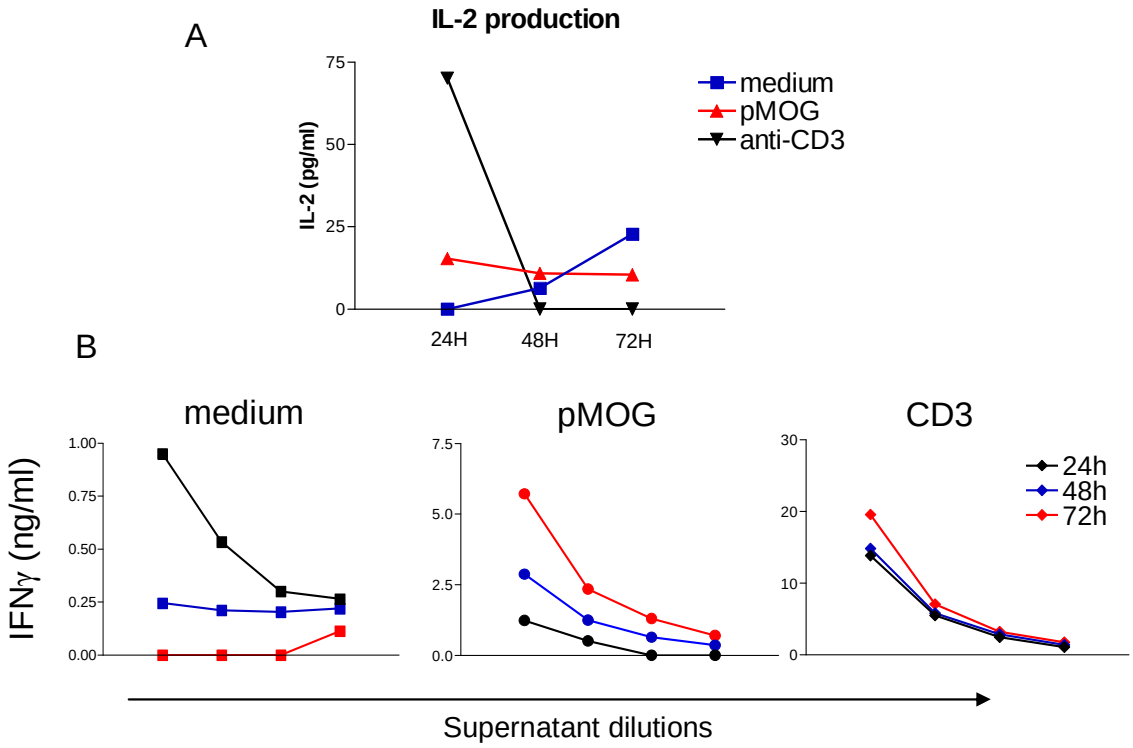


Figure 3.3: Production of cytokines by DLN of MOG-immunised C57BL/6 mice after in vitro re-stimulation. Supernatants from cultures in Fig. 3.2A were removed and assessed for cytokine levels by ELISA. A: IL-2 levels in supernatants of un-stimulated (blue line), pMOG (red line) and anti-CD3 stimulated (lack line) cells over the 72h culture. B: IFNγ levels in supernatants at 24h (black lines), 48 h (blue lines) and 72h (red lines) under each stimulation over two-fold dilutions. Data represents one of two repeat experiments with similar results.

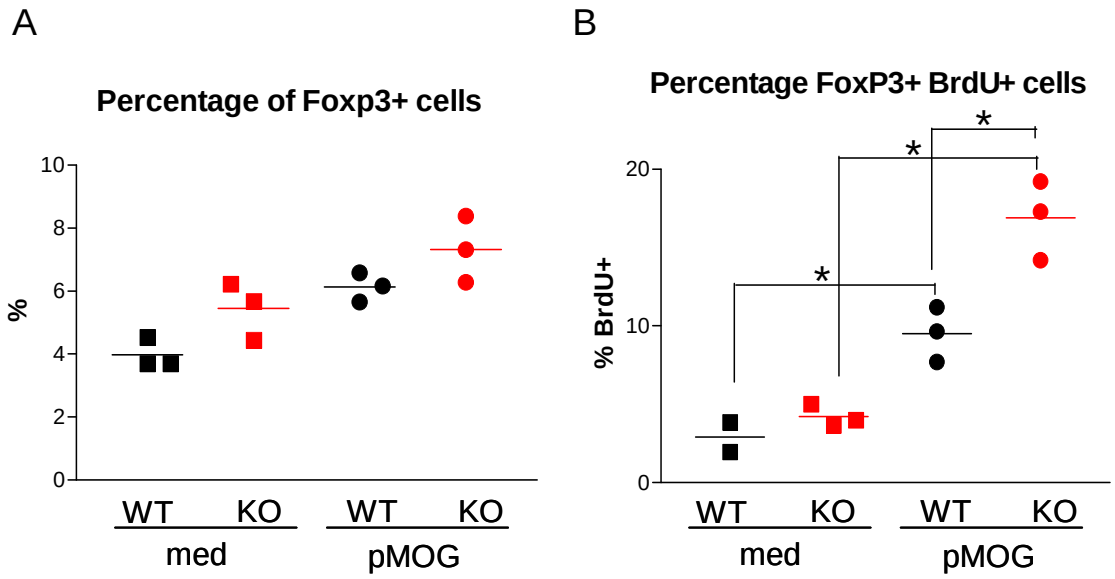


Figure 3.4: Foxp3+ cells from DLN of pMOG-immunised WT and MOGKO mice show low levels of proliferation to pMOG after 48h in vitro stimulation. DLN were removed from WT C57BL/6 (black) and MOGKO mice (red) were removed 10 days after immunisation with pMOG/CFA. Single cell suspensions were cultured with medium only or 10µM pMOG for 48h. BrdU was added after 36h of culture to assess proliferation by FACS at 48h. A: percentage of Foxp3+ cells after in vitro stimulation (gated on live CD4+ cells). B: Percentage of Foxp3+BrdU+ cells in each culture. Data points show triplicate cultures of pooled samples between groups. * $p<0.05$. Data represents one of four repeat experiments with similar results.

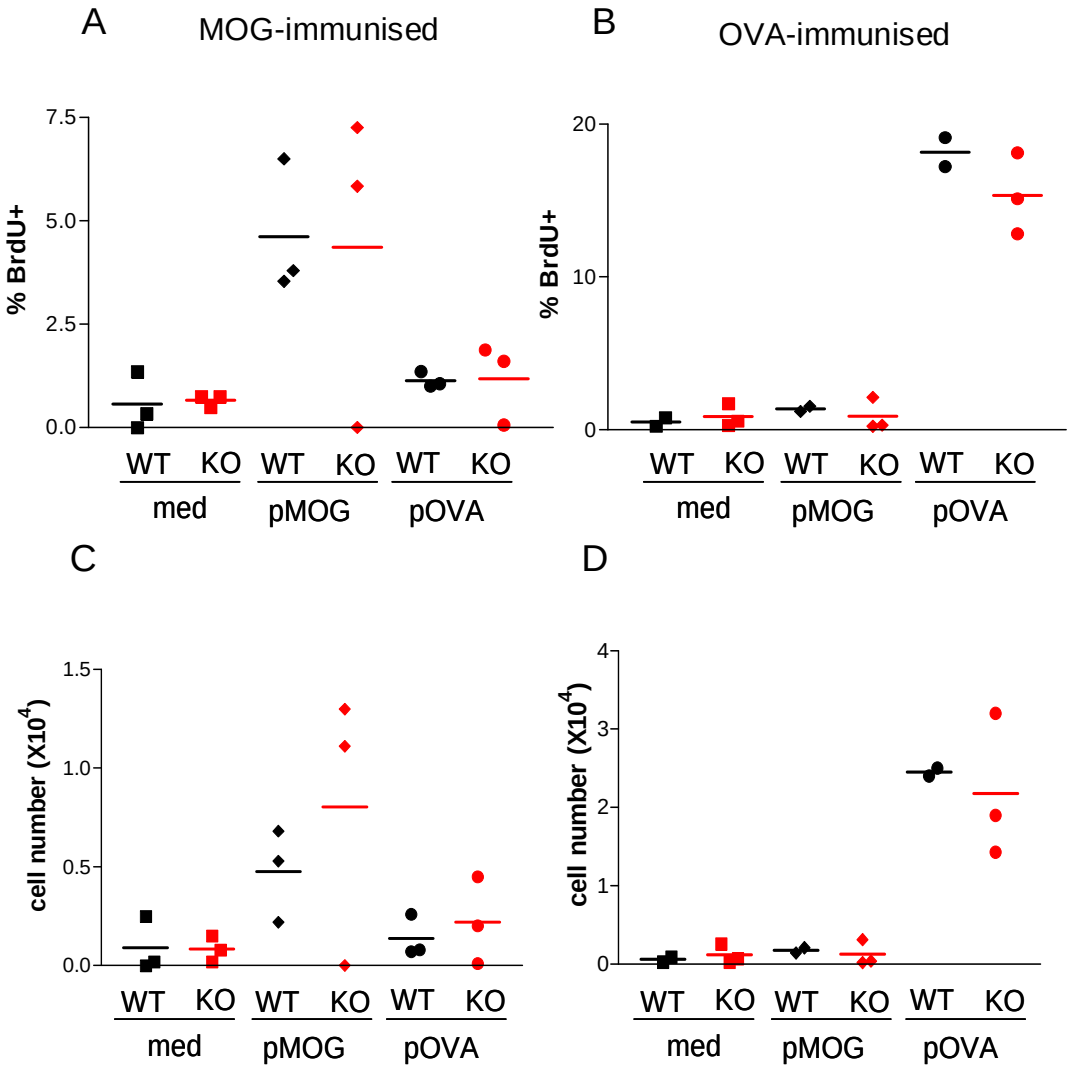


Figure 3.5: Foxp3+ cells from MOG-immunised MOGKO mice show enhanced proliferation compared to WT Foxp3+ cells in response to pMOG. DLN were removed from DLN of MOG-immunised (left panel) or pOVA-immunised (right panel) WT C57BL/6 (black) and MOGKO mice (red). Single cell suspensions were cultured with medium onle, pMOG or pOVA for 48h, with BrdU added for the final 15h of culture to assess proliferation of Foxp3+ cells by FACS. Top panels: Percentage of BrdU+Foxp3+ in A: pMOG immunised mice and B: pOVA immunised mice in response to in vitro restimulation with peptide. C and D: As above, shown as cell numbers. Data points show triplicate cultures of pooled samples between groups. Data represents one of three repeat experiments with similar results.

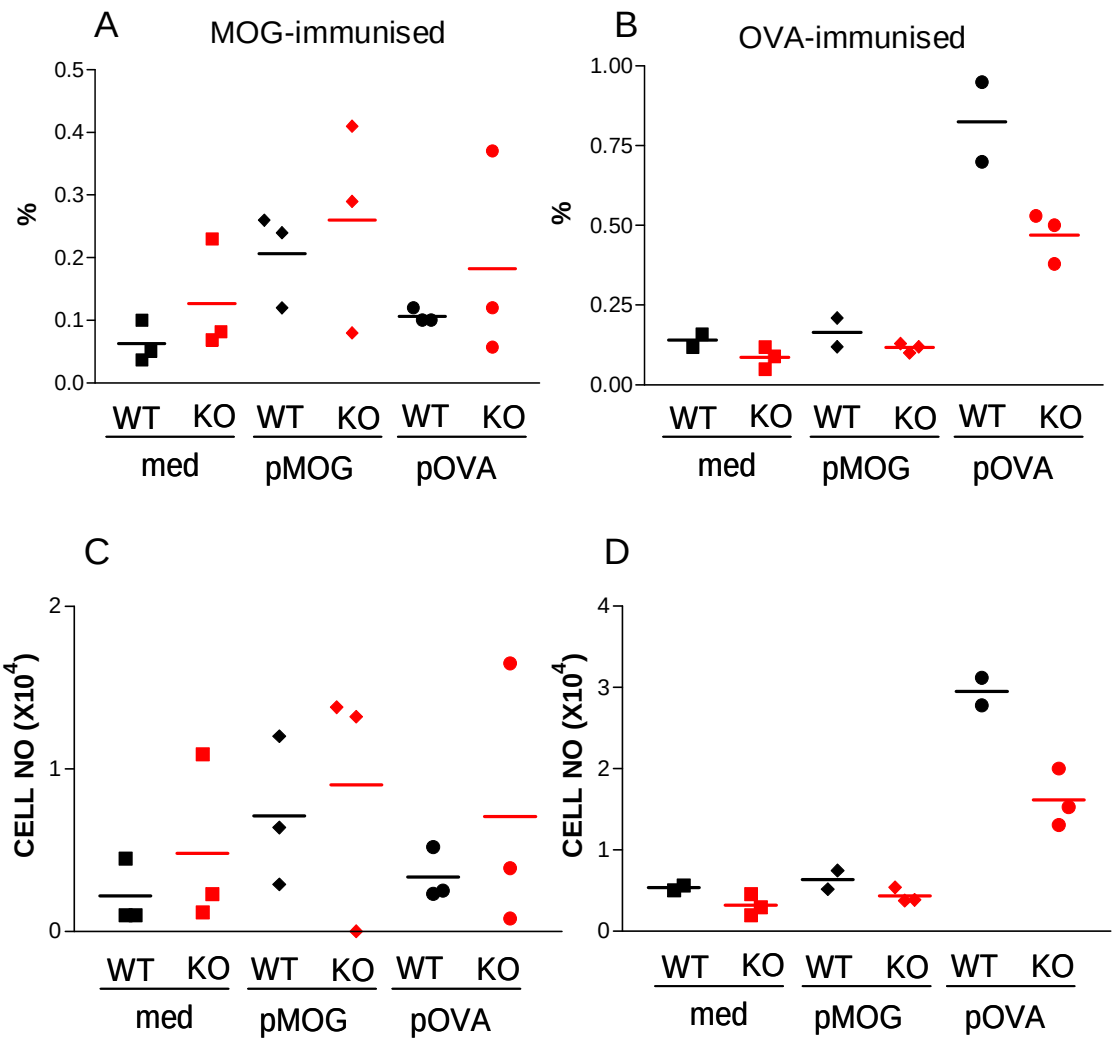


Figure 3.6: Foxp3- cells from pMOG or pOVA immunised WT and KO mice proliferate to the immunising antigen in vitro. DLN were removed from DLN of MOG-immunised (left panel) or pOVA-immunised (right panel) WT C57BL/6 (black) and MOGKO mice (red). Single cell suspensions were cultured with medium onle, pMOG or pOVA for 48h, with BrdU added for the final 15h of culture to assess proliferation of Foxp3- cells by FACS. Top panels: Percentage of BrdU+Foxp3- in A: pMOG immunised mice and B: pOVA immunised mice in response to in vitro restimulation with peptide. C and D: As above, shown as cell numbers. Data points show triplicate cultures of pooled samples between groups. Data represents one of three repeat experiments with similar results.

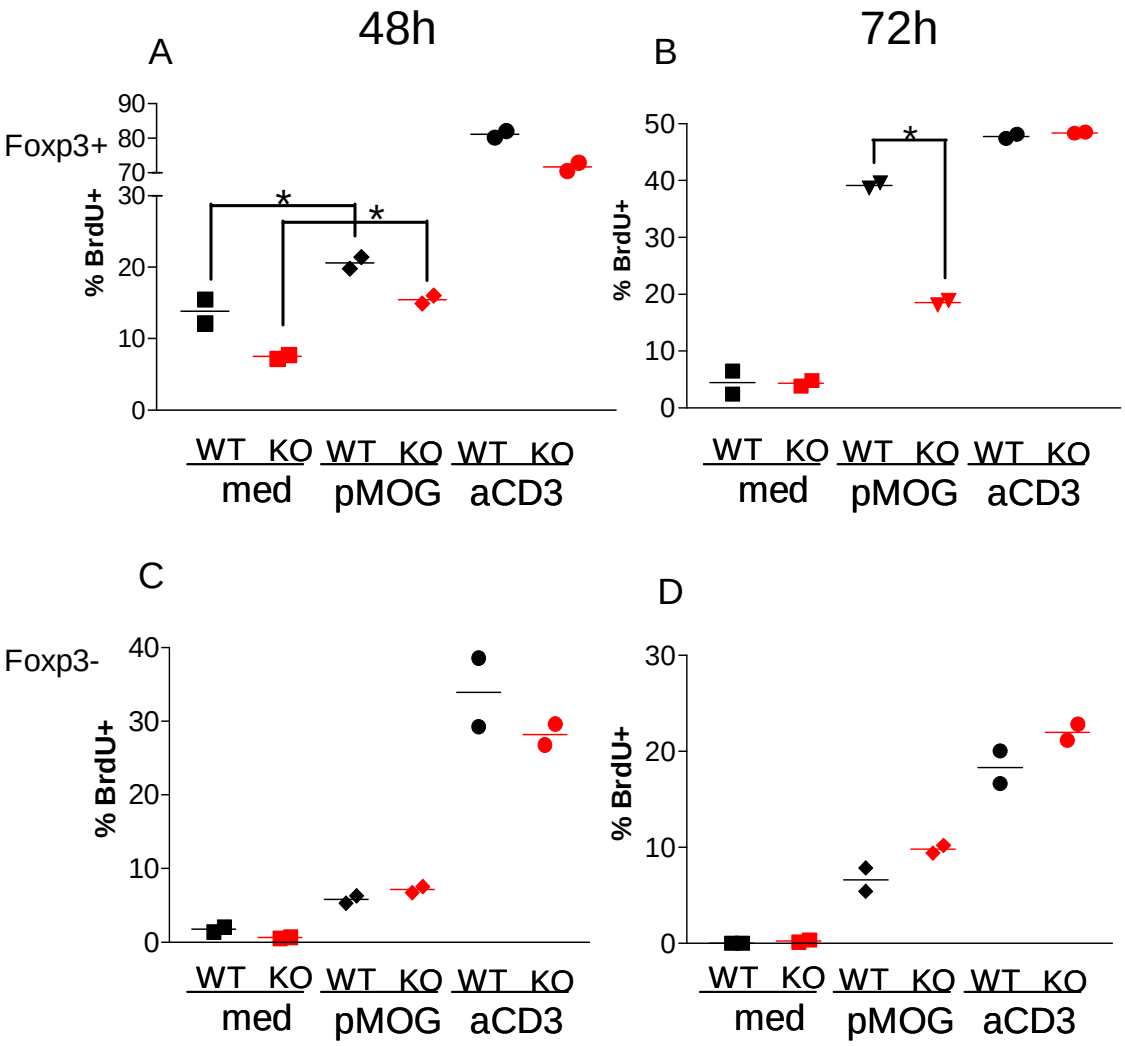


Figure 3.7: Sorted CD4+CD25+ cells from WT and MOGKO mice immunised with pMOG show proliferation in response to pMOG re-stimulation in vitro that is not accompanied by Foxp3- proliferation. DLN cells from MOGKO and WT mice were sorted into CD4+CD25+ populations by MACS and FACS sorting were stimulated with irradiated splenocytes from Thy1.1+C57Bl/6 mice alone or plus pMOG (10μM) for 48H and 72H. BrdU was added for the final 12h of culture and Foxp3+ and Foxp3- BrdU incorporation assessed by FACS. BrdU incorporation shown as a percentage of: A and B: Thy1.1-CD4+Foxp3+ cells and C and D: Thy1.1-CD4+Foxp3- cells. * = $p<0.05$. Data shows one of three repeat experiments with reproducible results.

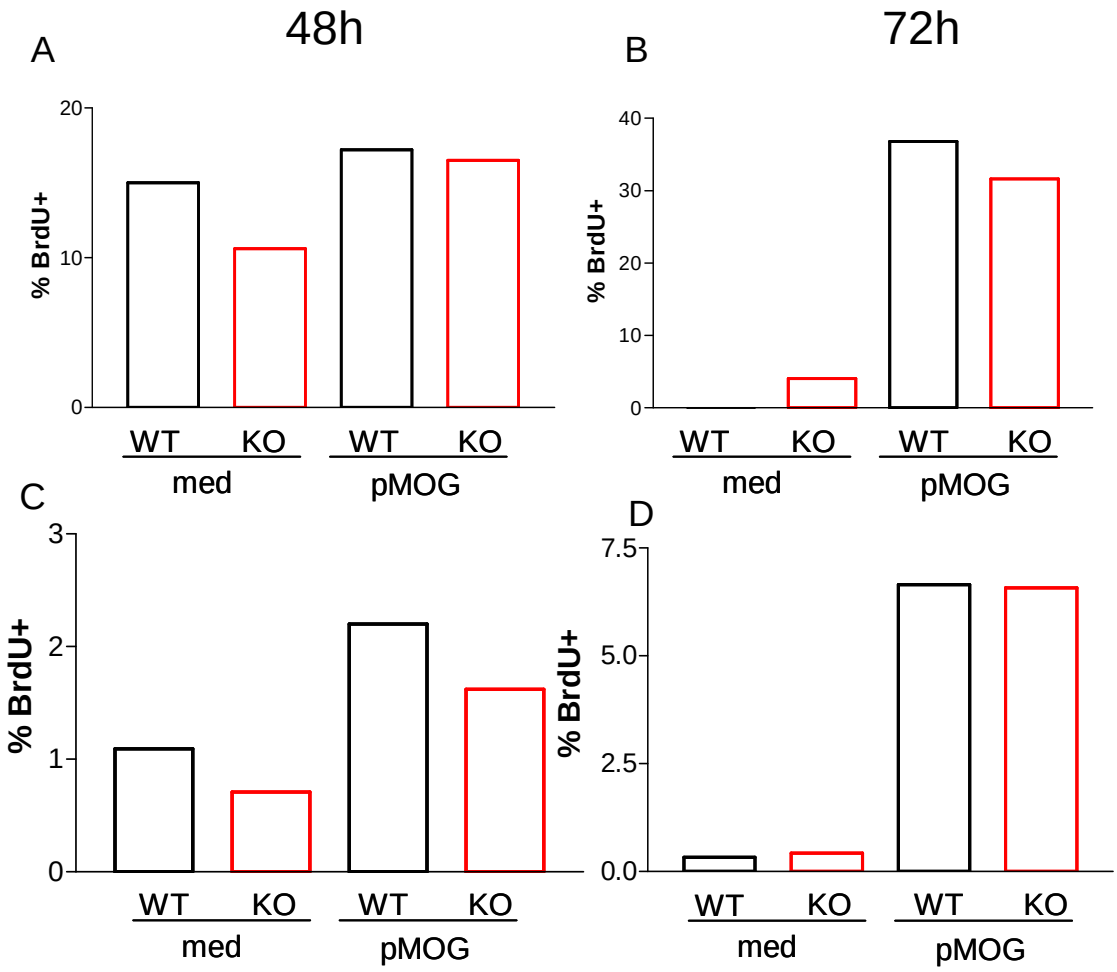


Fig 3.8: The proliferation of Foxp3+ cells from WT and MOGKO mice in response to pMOG is not affected when co-cultured with Foxp3- T cells. CD25+ cells were cultured at a 1:3 ratio with sorted CD25- cells and irradiated splenocytes from Thy1.1+ C57BL/6 mice as APC. Cells were stimulated with medium alone or 10µM pMOG for 48H and 72H. A and B: BrdU shown as a percentage of live CD4+Foxp3+ cells from WT (black bars) and MOGKO (red bars). C and D: Gated on CD4-Foxp3- cells. * = $p<0.05$. Bars represent individual wells of pooled samples, showing one of three separate experiments with similar results.

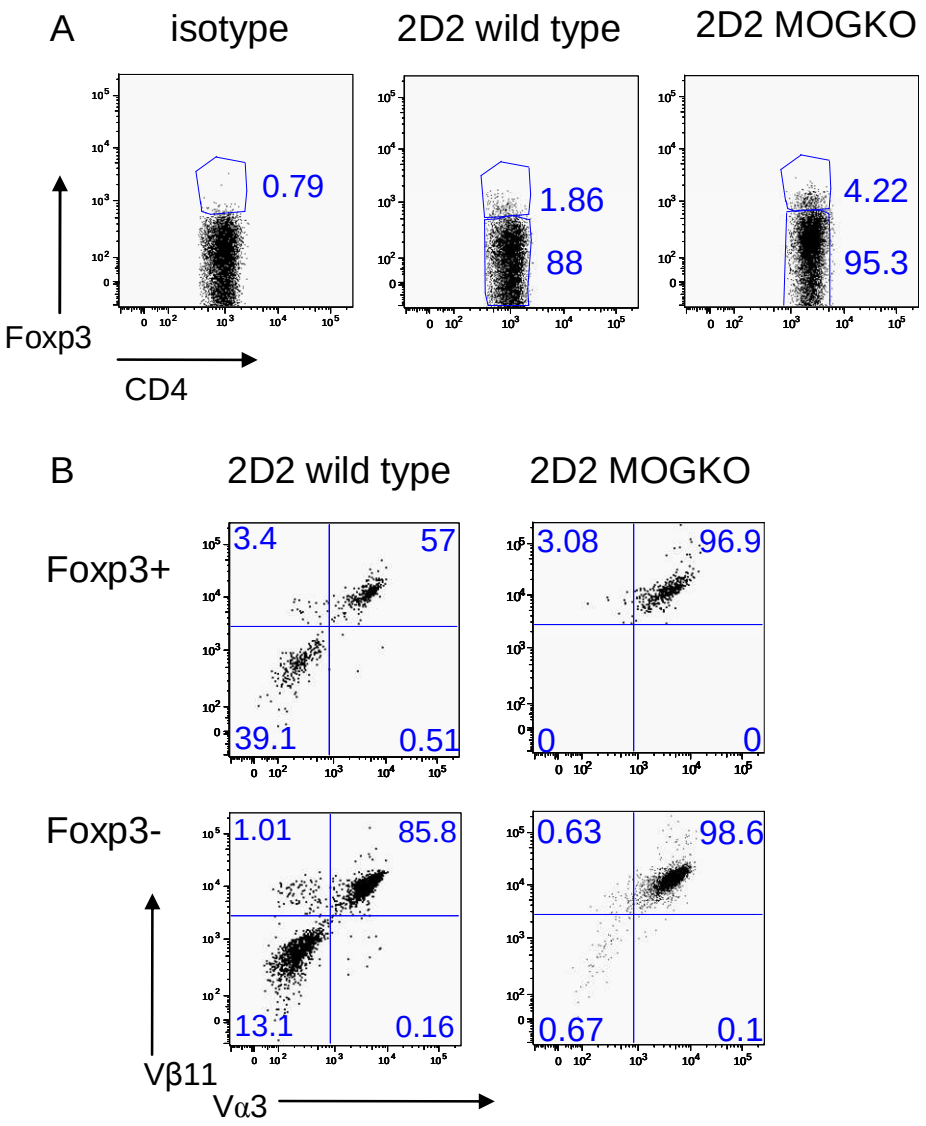


Figure 3.9: Naïve 2D2 WT vs 2D2 MOGKO transgenic TCR expression in Foxp3+ and Foxp3- populations in the spleen. A: Naïve lymphocytes from 2D2 WT or 2D2MOGKO mice assessed for Fop3-expression via FACS, gated on live CD4+ cells. Isotype control = IgG2a. B: Levels of transgenic TCR expression on WT and MOGKO 2D2 transgenic mice. Top panels: CD4+Foxp3+ cells, bottom panels: CD4+Foxp3- cells. Data show one representative plot of three individual mice per group. Data shows one of two separate experiments with similar results.

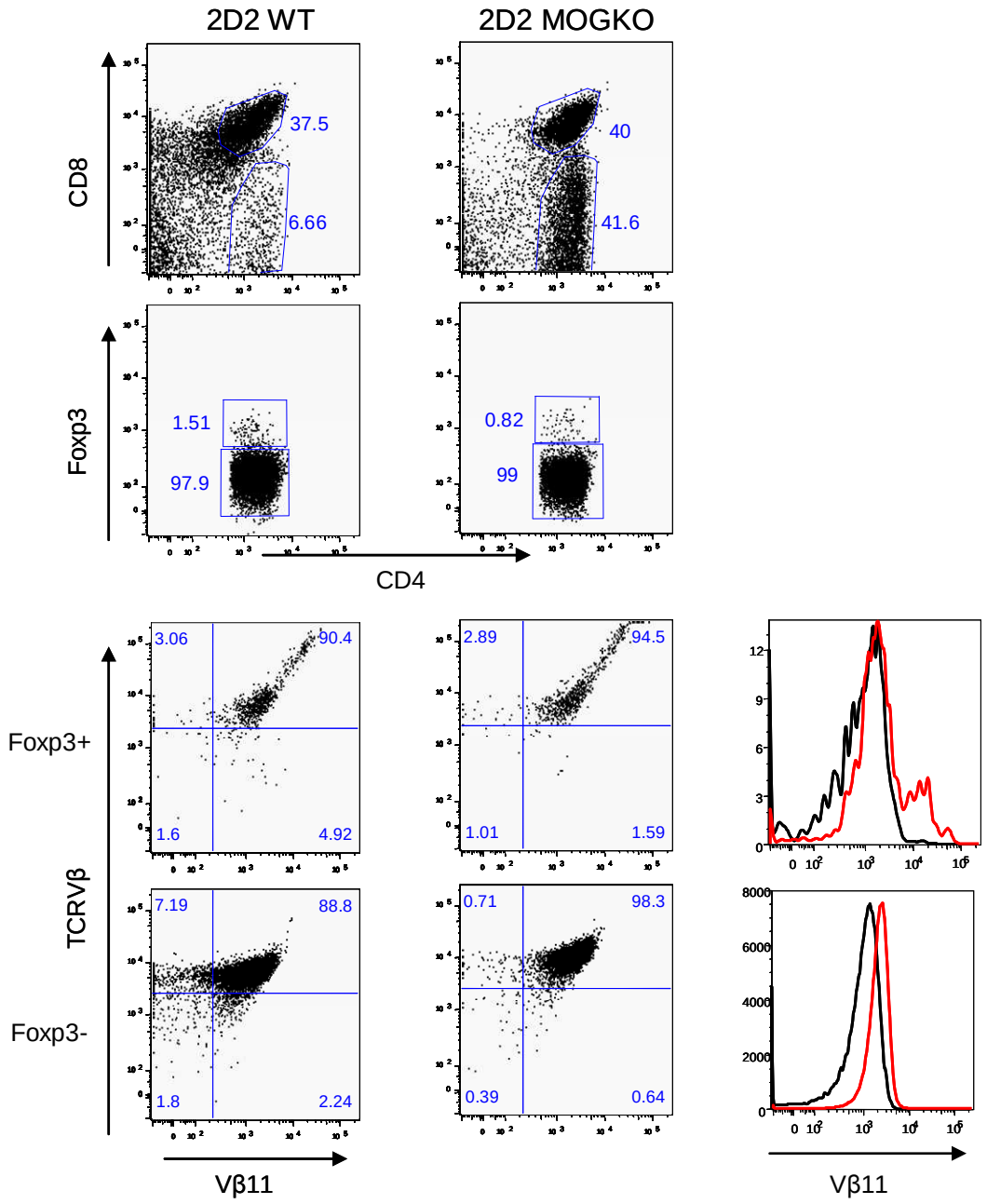


Figure 3.10: Naïve 2D2 WT vs 2D2 MOGKO transgenic TCR expression in Foxp3+ and Foxp3- populations in the thymus. A: Thymocytes from naïve WT and MOGKO 2D2 mice were removed and assessed for expression of CD4 and CD8 (top panels) and Foxp3 expression in CD4 single positive populations (bottom panels). B: Foxp3+ cells (top panels) and Foxp3- cells (bottom panels) were assessed for the expression of the transgenic TCR using Vβ11 antibody and for the expression of an alternative TCR by pan TCRVβ staining. C: Histograms showing the expression of Vβ11 expression on Foxp3+ (top panel) and Foxp3- cells(bottom panel), WT = black line, MOGKO = red line. Data representative of three individual mice assessed.

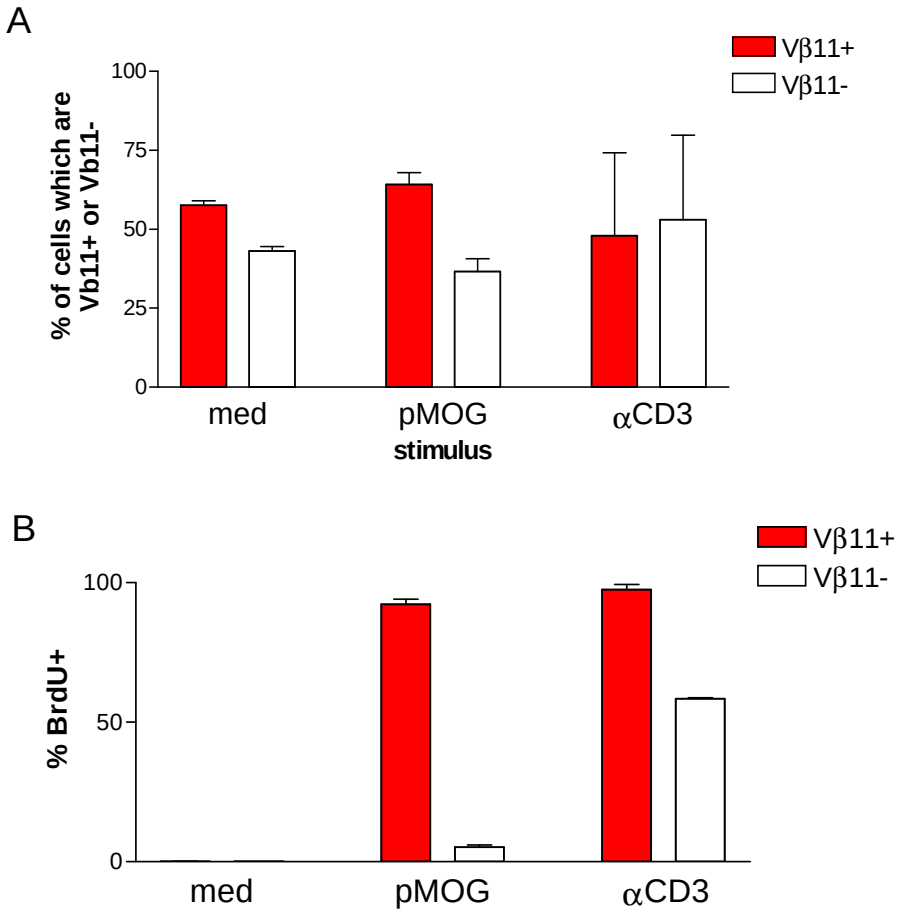


Figure 3.11: Only 2D2 cells expressing the transgenic (Vβ11+) TCR respond to pMOG in vitro. CD4+CD25+ cells were sorted from splenocytes of WT 2D2 mice and stimulated with pMOG (10µM) or anti-CD3 (1µg/ml) in vitro. BrdU incorporation in Vβ11+ and Vβ11- populations as assessed by FACS at 48h. A: Percentage of CD4+Vβ11+ (open bars) and CD4+Vβ11- cells (filled bars) after 48h in vitro stimulation. B: Percentage BrdU+ cells in Vβ11+ (red bars) and Vβ11- cells (open bars) after 48h in vitro stimulation. Error bars based on triplicate wells of each stimulated culture.

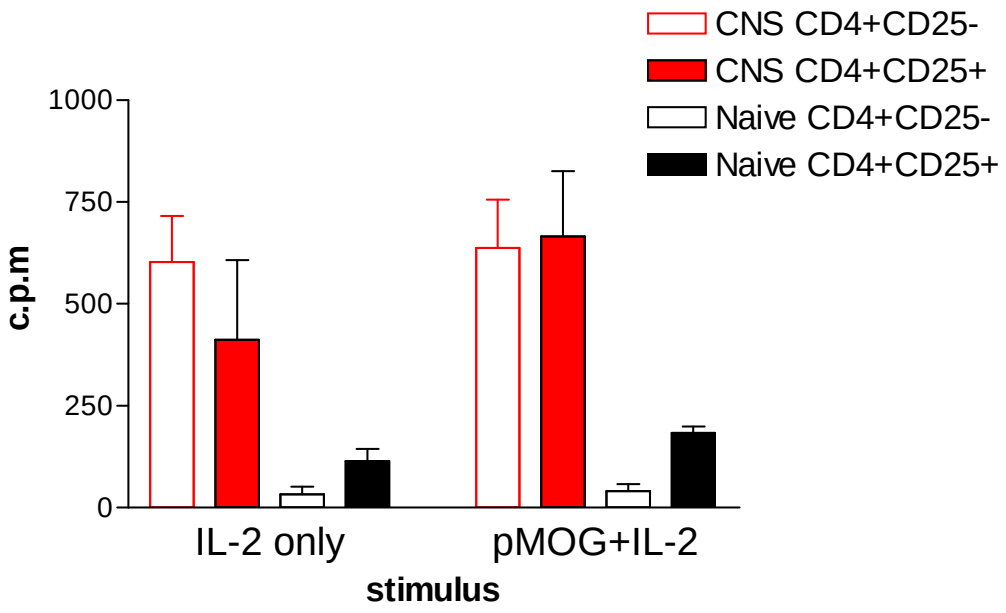


Figure 3.12: Proliferation of naïve and CNS sorted CD4+CD25+and CD4+CD25- populations to pMOG and IL-2 as determined by ^{3}H-thymidine incorporation. Splenocytes from naïve C57BL/6 mice or lymphocytes isolated from the CNS of mice which have undergone pMOG/CFA-induced EAE for 16 days were sorted into CD4+CD25+ or CD4+CD25- populations to >90% purity. Cells were cultured @ 1x10^{4} cells/well with 5x10^{5} irradiated splenocytes from C57BL/6 mice +/- pMOG (10µg/ml) + IL-2 (100U/ml) for 72h. Tritiated thymidine was added to the cultures and assessed for thymidine incorporation after 18h. Error bars show mean +/- S.D. of triplicate wells of pooled samples between groups, data representative of one of three individual experiments with similar results.

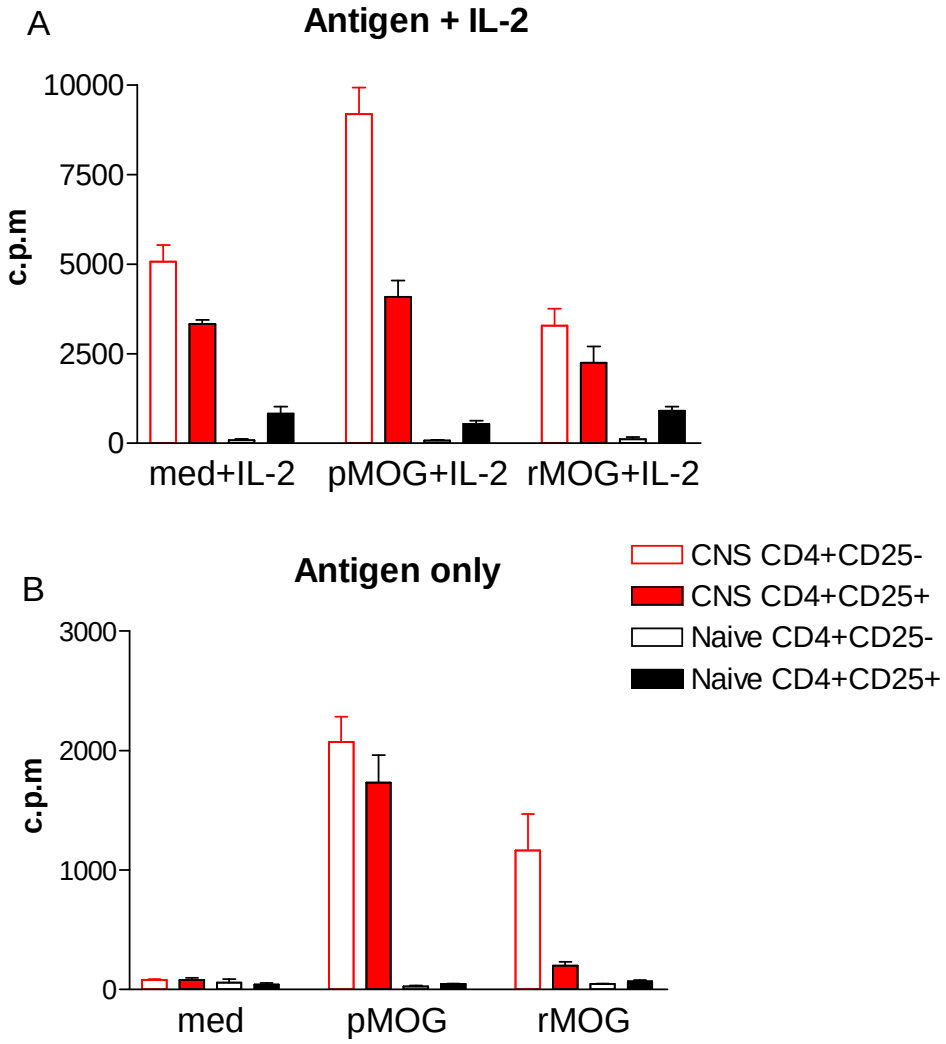


Figure 3.13: IL-2 induced proliferation masks antigen-induced proliferation as measured by thymidine incorporation. Splenocytes from naïve C57BL/6 mice or Lymphocytes isolated from the CNS of mice which have undergone pMOG/CFA-induced active EAE for 16 days were sorted into CD4+CD25+ or CD4+CD25- populations to >90% purity. Cells were cultured @ $1x10^4$ cells/well with $5x10^5$ irradiated splenocytes +/- pMOG (10µg/ml). A: cells cultured with APC, antigen and IL-2 (100U/ml). B: cells cultured with APC + antigen in the absence of exogenous IL-2. Cultures were incubated for 72h. Tritiated thymidine was added for the final 18H of culture before detection of thymidine incorporation. Error bars show mean +/- S.D. of triplicate wells of pooled samples between groups, data representative of one of three individual experiments with similar results.

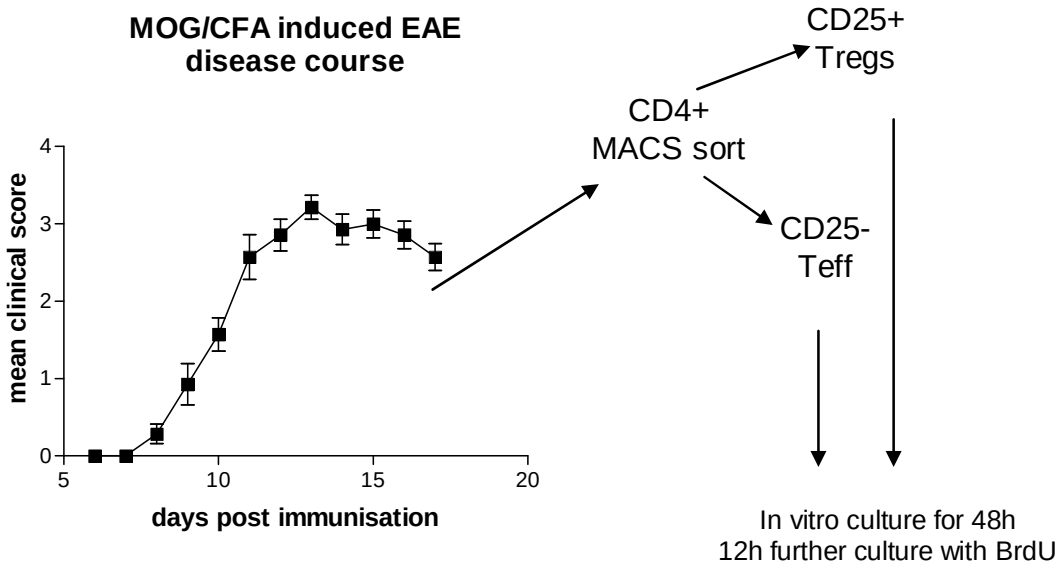


Figure 3.14: Schematic representation of BrdU incorporation assay. Cells isolated from CNS and sorted CD4+ (MACS) CD25+/CD25- (Aria). CD25+ sorted to 94% purity. CD25- sorted to 97% purity. Cultured at $3x10^5$/ml, 100µl in 96 well round bottom plate. Irradiated Thy1.1 APC $1x10^7$/ml, 50 µl/well, IL-2 (when added) at 0.1ng/ml final.

Table 3.1: Antigens and concentrations used for in vitro stimulation of sorted populations.

antigen	Conc.
pMOG	10 µg/ml
rMOG	1 µM
rMBP	1 µM
Bov. MBP	2 µM
rm HSP60	20 µg/ml
rMtb HSP60	20 µg/ml
PPD	10 µg/ml

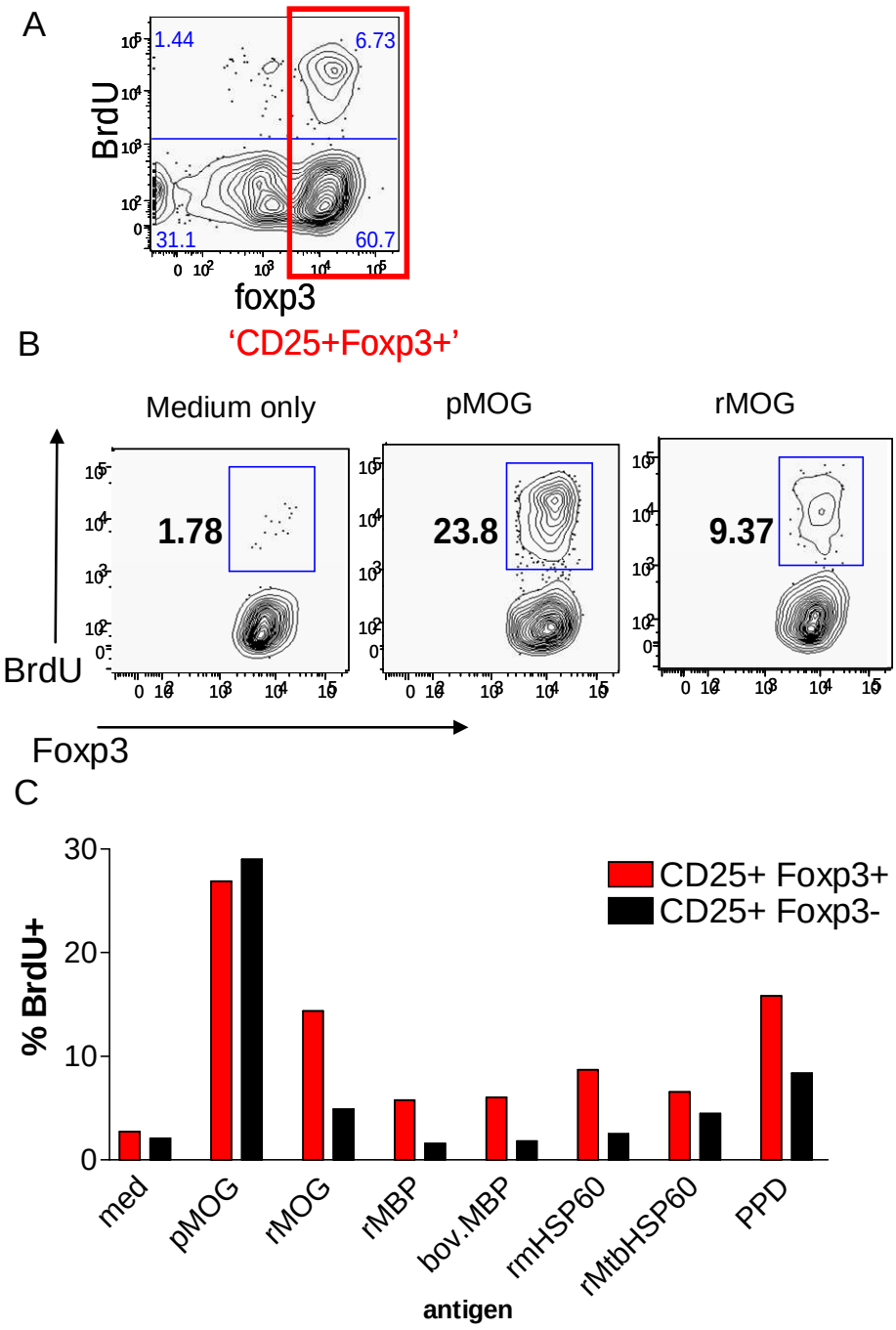


Figure 3.15: Foxp3+ cells isolated from the CNS show BrdU incorporation in response to the immunising antigen. A: FACS plot demonstrating the post-in vitro culture Foxp3 vs BrdU plot of CD25+ sorted cells stimulated with rMOG in vitro. B: BrdU incorporation of cells from CD25+ cells in un-stimulated, pMOG stimulated and rMOG stimulated cultures, gated on CD4+ Foxp3+ cells. C:CNS derived CD4+CD25+ sorted Foxp3+ cell (red bars) and Foxp3- cell (black bar) % BrdU incorporation in response to indicated antigen. Bars show individual wells of pooled samples between groups. Data shows one of two repeat experiments with similar results.

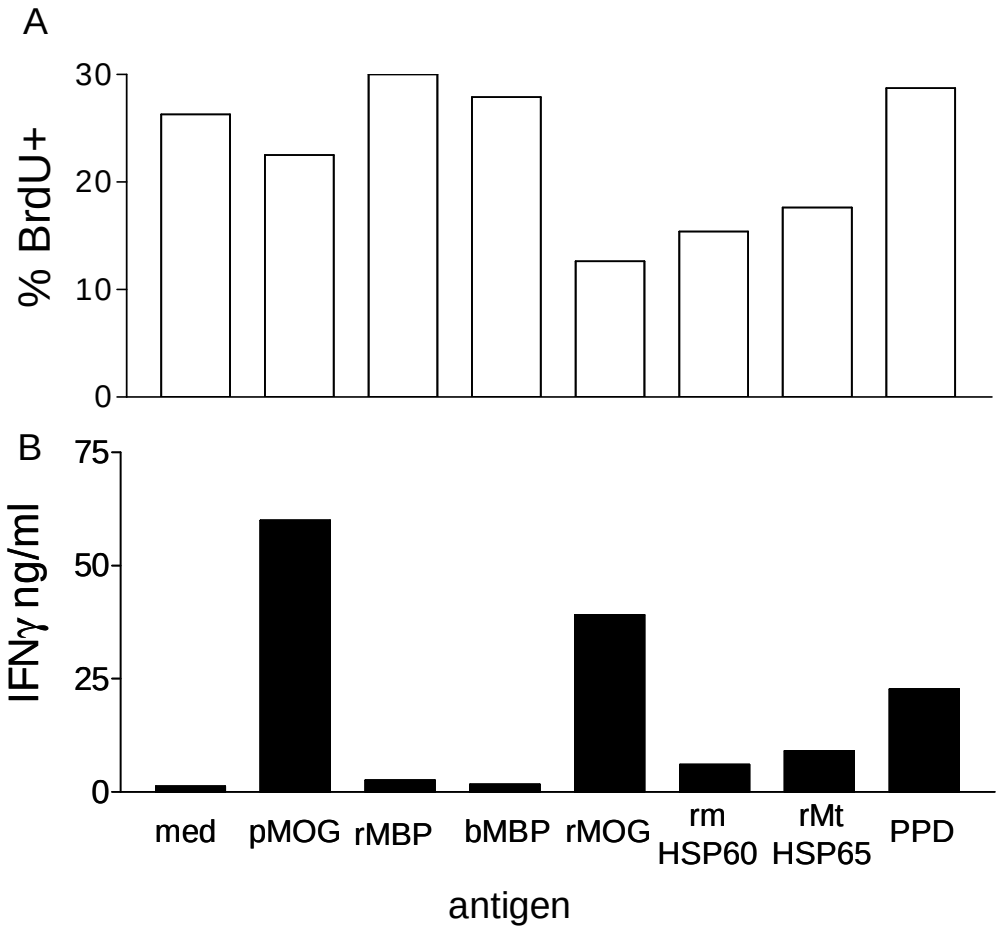


Figure 3.16: CNS-derived Teff cell BrdU incorporation and IFNγ production after in vitro stimulation. A: CNS derived CD4+CD25- sorted BrdU incorporation in response to indicated antigen. B: Supernatant IFNγ levels as detected by ELISA in CD4+CD25- populations (from figure A) after 72h in vitro stimulation with indicated antigen. Bars show individual wells of pooled samples between groups. Data shows one of two repeat experiments with similar results.

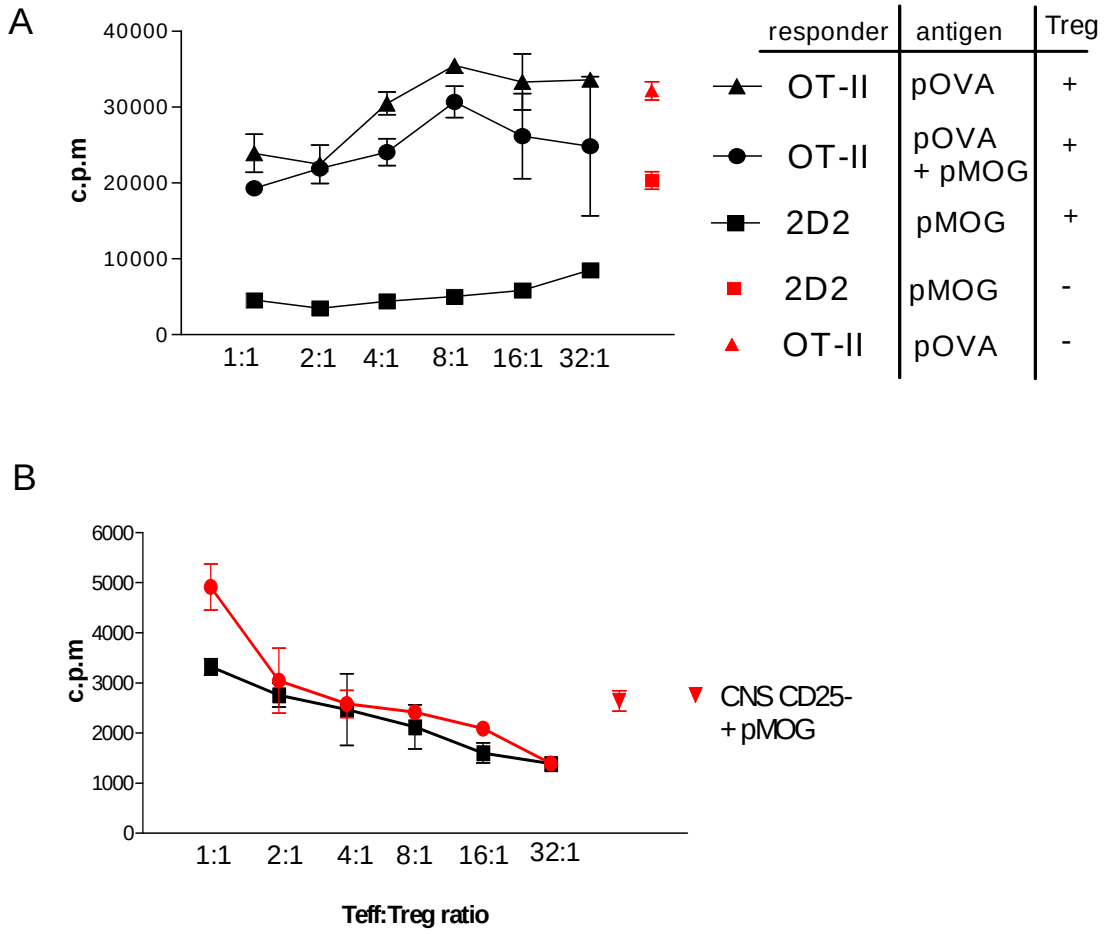


Figure 3.17: CNS Tregs can suppress proliferation of naïve, pMOG-reactive TCR transgenic effector T cells, but not CNS-derived effector cells. CNS-derived Tregs were cultured at the indicated ratio with purified CD25- cells ($2x10^4$) from A: 2D2 TCR transgenic mice + pMOG (squares), OTII TCR transgenic CD25- + pOVA (triangles) or OT-II CD25- cells + pMOG + pOVA (circles) and B: CNS derived Tregs (red bars) and naïve Tregs (black bars) were cultured with CD25- cells sorted from CNS and stimulated with pMOG. Irradiated slenocytes from niave C57BL/6 mice were used as APC in all cultures. Dotted line represents average c.p.m. of responders alone in culture with antigen. Error bars show mean +/- S.D. of triplicate wells of pooled samples between groups. Data is representative of two independent experiments with similar results.

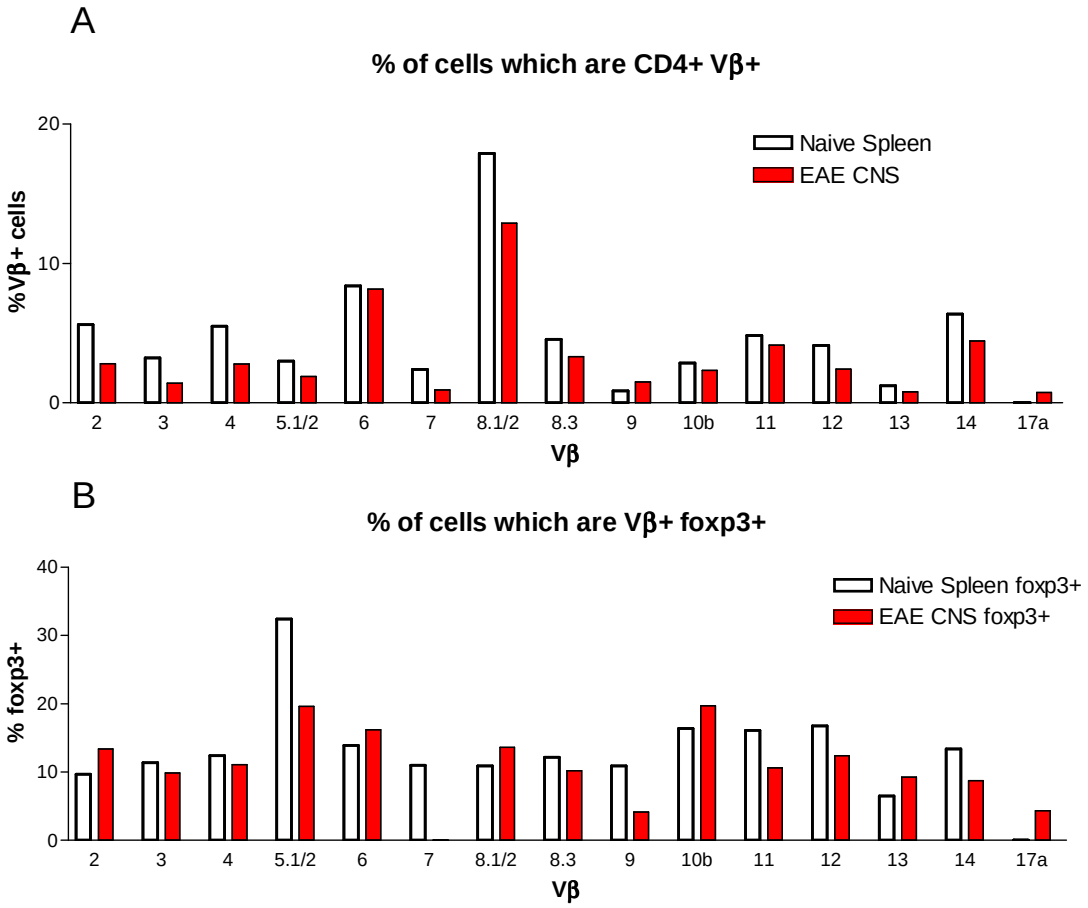


Figure 3.18: TCR Vβ analysis does not show skewing of TCR usage of Tregs from the inflamed CNS vs. naïve splenocytes. Lymphocytes were isolated from the CNS of mice which had undergone pMOG/CFA immunisation 16 days earlier or from the spleen of naïve C57BL/6 mice. Cells are gated on live CD4+ from CNS (red bars) or naïve splenocytes (black bars) and assessed for A: TCR Vβ expression and B: Foxp3-expression within each Vβ+ population via FACS. Data represents one of three repeat experiments showing similar results.

4 Therapeutic use of myelin basic protein-reactive Tregs in CNS autoimmune disease

4.1 Introduction

It has been shown using animal models that transfer of Tregs can suppress inflammatory disease. A number of studies have highlighted that antigen-specific, disease relevant Tregs are much more efficient at suppressing a model of diabetes, even at low cell numbers (Tang et al., 2004b; Tarbell et al., 2004). If the antigen reactivity of Tregs is essential to activate Treg mediated suppression it will be crucial to target the expansion of CNS-relevant Tregs in EAE and MS to ameliorate disease.

Some inflammatory disorders in mice, such as EAE and models of diabetes, are associated with a diverse T cell repertoire, recognising more than one antigen (Acha-Orbea, 1991; Mallone and van Endert, 2008). Furthermore, the disease initiating antigen is not always known. In EAE, as destruction of the myelin sheath occurs this mediates the release of new proteins and peptides which were previously ignored by the immune system (Vanderlugt and Miller, 1996). The release of inflammatory mediators in the CNS enhances antigen presentation by CNS resident cells and infiltrating APCs by inducing the up-regulation of MHC and co-stimulatory molecules (Becher et al., 2006). This can allow the activation of T cells against newly emerging epitopes of the same and distinct antigens resulting in epitope spreading. This phenomenon was described in the B10.PLxSJL model using MBP(Ac1-9)-induced EAE (Lehmann et al., 1992), where the immune response was shown to spread to other epitopes of MBP as the disease progressed (intramolecular spreading). It had also previously been reported that spreading could occur to epitopes of PLP after immunisation with MBP (intermolecular spreading) (Perry et al., 1991). Furthermore, the initiation of disease against PLP(139-151) resulted in the emergence of T cells reactive against other PLP epitopes, including PLP(178-191) and MBP epitopes (Vanderlugt et al., 2000). The fact that over the course

of disease distinct repertoires of T cells arise that are reactive against different myelin epitopes, suggests that epitope spreading contributes to the relapses associated with demyelinating diseases (Tuohy et al., 1999; Vanderlugt et al., 2000).

This phenomenon presents a problem in antigen-specific therapy, as tolerance to one antigen will not necessarily induce tolerance to other antigens. However, previous studies using oral and nasal peptide administration have suggested that tolerance induced to one myelin epitope can induce bystander suppression against distinct myelin antigens, suggesting that antigen-specific therapy may be beneficial in this setting (Anderton et al., 1998; Metzler and Wraith, 1993; Miller et al., 1991)

The results in this chapter describe experiments that aimed to target the expansion of myelin-reactive Tregs both in vitro and in vivo. We have investigated the capacity of MBP-reactive Tregs to suppress both MBP(Ac1-9)- and PLP(139-151)-induced EAE to determine if specific targeting of antigen-reactive Tregs will be sufficient to overcome a disease that involves a diverse autoimmune T cell repertoire. These studies have important implications in the development of Treg-targeted peptide based therapy for the treatment of MS.

4.2 Results

4.2.1 Generation of sufficient Treg numbers by in vitro expansion

As has been discussed, isolation of sufficient antigen-specific Treg cell numbers is a difficult task. However, the potential to expand ex vivo isolated Tregs in vitro using TCR stimulation and IL-2 has now been shown in both mouse and human studies (Earle et al., 2005; Fisson et al., 2006; Tang et al., 2004b) and importantly these Tregs maintained their suppressive capacity in vitro and in vivo after the expansion protocol. Recent investigations have also suggested that expanded Tregs show an enhanced capacity to suppress (Chai et al., 2008). We therefore investigated the potential of myelin-reactive Tregs to be expanded in vitro, following their Foxp3-expression over the expansion period and assessed the suppressive capacity of the expanded Tregs in various models of EAE.

Prior to the generation of Foxp3-GFP reporter mice (Fontenot et al., 2005b), sorting of Tregs based on their Foxp3-expression was not possible due to its intracellular expression. However, in naïve mice, sorting of CD4+ cells based on expression of CD25 can isolate ~85% of all Foxp3+ cells (Sakaguchi et al., 1995). We therefore purified CD4+ cells via MACS sorting, followed by FACS-purification of CD25- (Teff) and CD25+ cells (Tregs) from spleen and lymph nodes of naïve Tg4 transgenic mice (MBP Ac1-9-reactive TCR). Cells were then cultured with anti-CD3/anti-CD28 coated beads plus high levels of IL-2 to overcome Treg cell anergy. In numerous attempts to generate highly pure Treg populations we consistently found that while the CD4+CD25+ population showed a high Foxp3+ fraction before expansion, this was dramatically reduced after 7 days of expansion (from ~85% pre-expansion to 20% post-expansion).

4.2.1.1 Sorting Tregs based on CD62L expression

It has been demonstrated that CD25+CD62L+ Foxp3+ Tregs have an enhanced suppressive capacity compared to CD25+CD62L- Tregs in vivo and maintained the expression of Foxp3 when expanded in vitro (Fu et al., 2004). This is in agreement with studies from our lab showing that sorting of Tregs based on high expression levels of CD62L generates a population of highly pure Foxp3+ cells (Leigh Stephens, personal communication). To test the ability to sort Tregs based on the expression of CD62L we purified CD4+ T cells from the spleen and LN of naïve Tg4 mice and sorted them into three groups based on their expression of CD25 and CD62L; CD25-, CD25+CD62L^{lo} and CD25+CD62L^{hi} (Fig. 4.1A). Each population was sorted to >93% purity and assessed for Foxp3 expression by FACS. Both the CD25+ populations (CD62L+ and CD62L-) showed high proportions of Foxp3+ cells, while only low numbers of CD25- cells expressed Foxp3 (Fig. 4.1B, left panel). The levels of Foxp3 expression in each population was assessed after 7 days of in vitro expansion using anti-CD3/anti-CD28 coated beads and IL-2 (Fig. 4.1B, right panel). The CD25- did not increase the proportion of cells expressing Foxp3 over the expansion period. While the CD25+CD62L^{lo} population showed a high proportion of Foxp3 expression before expansion (>80%), this was dramatically reduced after the 7 day culture, suggesting either out-growth of the contaminating 20% Foxp3- population, death of Foxp3+ cells or the loss of Foxp3-expression by some of the cells. The CD25+CD62L^{hi} population showed a higher proportion of Foxp3+ cells after sorting (>90%) and while this was reduced after the 7 day culture to ~70%, it suggested that Foxp3 expression was more stable in this population during in vitro expansion.

4.2.1.2 In vitro expanded Tregs suppress EAE induced with their cognate antigen

To assess the suppressive capacity of Tg4 Tregs in EAE, we sorted CD4+CD25+CD62L^{hi} cells from the spleen and LN of Tg4Ly5.1+ mice and expanded these cells in vitro. As a control, polyclonal CD4+CD25+CD62L^{hi} Tregs were also sorted and expanded from B10.PL mice. Tregs were routinely around 90% Foxp3+ in both Tg4 and B10.PL populations. 1x10^5 Tregs were transferred i.v. to B10.PL hosts 1 day prior to immunisation with MBP(Ac1-9)/CFA and assessed for clinical signs of EAE over a 30 day period (Fig. 4.2). Mice that received polyclonal Tregs did not show any reduction in disease incidence or severity compared to mice that received PBS alone (Fig. 4.2). However, mice which received MBP-reactive Tregs had a reduced incidence of disease of 50% in Tg4 Treg transfer group vs. 100% in PBS only group (Fig. 4.2). Those mice developing clinical signs in the group that had received Tg4 Tregs had less severe disease compared to the PBS treated group with a mean maximal clinical score of 1.5 and 3.75 respectively. These data demonstrated that a very small number of antigen-specific Tregs could efficiently reduce EAE when given prior to disease induction, while polyclonal Tregs could not.

4.2.2 Expanding Tregs in vivo using peptide-based therapy

The ultimate aim of antigen-specific therapy is to induce antigen-specific unresponsiveness in vivo. It has long been established that the administration of soluble peptides together with an adjuvant will induce immune activation and result in a sustained and productive immune response. However, administration of antigen in the absence of adjuvant leads to abortive immune activation, resulting in antigen-specific unresponsiveness, or tolerance (Dresser, 1962). Many tolerogenic protocols involve the administration of peptides from disease-relevant antigen(s), also termed peptide therapy. This has been shown to be successful in models of autoimmunity, inflammation and

allergy and represents a potential therapy that could be translated to human disease (Anderton, 2001; Larche and Wraith, 2005). By using adoptive transfer models, some studies have demonstrated clonal elimination of most antigen-reactive T cells after peptide administration (Critchfield et al., 1994; Liblau et al., 1996). The few remaining antigen-reactive T cells could not be stimulated by their cognate antigen and were considered anergic (Pape et al., 1998). Other studies have documented the generation of T cells that produce high levels of IL-10 (Burkhart et al., 1999; Sundstedt et al., 2003), perhaps reflecting Tr1-like regulatory cells (see introduction). However, only a few studies have demonstrated the induction or expansion of Foxp3+ regulatory T cells. These studies used high levels of TGFβ and/or IL-2 to generate Foxp3+ T cells in vitro (Selvaraj and Geiger, 2007; Zheng et al., 2007a) or used low dose, antigen administration (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005) to target Foxp3+ cell conversion/expansion in vivo in the absence of inflammation. We therefore attempted to target the expansion of antigen-reactive Foxp3+ T cells therapeutically in EAE using CNS-derived peptide administration.

To track the expansion of Tregs in vivo, we used adoptive transfer of CD4-purified Tg4Ly5.1+ cells to Ly5.2+ B10.PL host mice. The Tg4 model is particularly useful for the study of peptide based tolerance as the TCR and MHC binding properties of the MBP (Ac1-9) peptide have been extensively studied. Residues 3 and 6 of the nonapeptide have been shown to be interaction TCR determinants for I-Au restricted T cells (Wraith et al., 1989). Wild-type MBP (Ac1-9) has been shown to bind poorly to I-Au, such that the half life of peptide binding cannot be determined in vitro (Fairchild et al., 1993; Liu et al., 1995). This has been attributed to the lysine at positon 4 of the peptide which interacts unfavourably with the hydrophobic pocket in the MHC binding groove (Pearson et al., 1999). Substitutions for lysine at position 4 have been shown to affect the binding affinity of the peptide to the MHC, with the more hydrophobic 4Tyrosine peptide showing binding affinities of 100,000 fold higher than the WT peptide (Fairchild et al., 1993). Use of this altered peptide ligand (APL) showed it to have enhanced tolerogenic properties, suppressing EAE to a greater extent than the WT

4Lys peptide (Liu and Wraith, 1995). The persistence of Ac1-9(4Tyr) for long periods in vivo makes this a unique peptide that will provide continual stimulation of peptide-reactive T cells; we therefore reasoned that this APL may induce the upregulation of Foxp3 or preferential expansion of Foxp3+ cells in vivo.

4.2.2.1 Treatment with Ac1-9(4Tyr) maintains a population of MBP-reactive T cells which are tolerant to in vitro re-stimulation

In order to assess the capacity of Ac1-9(4Tyr) to expand Foxp3+ T cells in vivo, CD4+ cells were sorted from Tg4Ly5.1+ cells to ~98% purity, with approximately 3% of cells expressing Foxp3+ (Fig 4.3A). 2x106 cells were then transferred to B10.PL (Ly5.2+) mice one day before peptide treatment. The dose of Ac1-9(4Tyr) which would be optimal for expansion of Foxp3+ cells was unknown, therefore mice were given either 10μg (low dose) or 100μg (high dose) of Ac1-9(4Tyr). As controls, mice were given PBS or 10μg Ac1-9(4Tyr) plus 10μg LPS i.v.. After 6 days spleen and lymph nodes were harvested to determine the proportion of Tg4 cells and the levels of Foxp3-expression (Fig 4.3B and C). Mice that had received 100μg Ac1-9(4Tyr) maintained a greater proportion of Tg4 cells than 10μg Ac1-9(4Tyr) or PBS treated mice, demonstrating that high dose peptide treatment had expanded the Tg4 populations (Fig. 4.3B). Mice which had been treated with Ac1-9(4Tyr) and LPS showed a greater expansion of Tg4 cells, as these cells should have been fully activated by the adjuvant and expanded in response to the antigenic stimulation (Fig. 4.3B).

The levels of Foxp3 expression within each of the Tg4 populations was then assessed (Fig 4.3C, top panels). Mice which had been treated with 10μg Ac1-9(4Tyr) or Ac1-9(4Tyr) plus LPS did not show enhanced proportions of Foxp3+ cells in the Tg4 transfer population (Fig. 4.3C). Mice that had received 100μg Ac1-9(4Tyr) showed an increased proportion of Foxp3+ cells compared to 10μg Ac1-9(4Tyr)- or Ac1-9(4Tyr) plus LPS-treated groups, and compared to the proportion of Foxp3+ cells in the initial transfer

population. However, the difference in Foxp3 expression was not vast, only increasing by 1-2%. It is also notable that the level of Foxp3 staining within the PBS treated group was similar to the 100μg Ac1-9(4Tyr) treated group, and therefore represents a population of Foxp3+ within the normal range for naïve mice and is not enhanced by peptide treatment. The proportion of Foxp3+ cells in host populations was also only enhanced by ~2% in any of the treatment groups compared to PBS-treated controls (Fig. 4.3C, bottom panels).

To assess the in vitro response of each group, splenocytes were stimulated in vitro with MBP(Ac1-9) and proliferation measured after 72h (Fig 4.3D). Mice that had been treated with 100μg Ac1-9(4Tyr) were unresponsive to in vitro re-stimulation with the WT peptide. Mice treated with 10μg Ac1-9(4Tyr) did not show reduced responsiveness to Ac1-9 compared to PBS treated mice, while mice that had received Ac1-9(4Tyr)+LPS demonstrated enhanced proliferation in vitro. These data demonstrated that treatment with 100μg Ac1-9(4Y) maintained a population of Tg4 cells in vivo that were tolerant to in vitro re-stimulation with MBP(Ac1-9) but only showed a minimal increase in the proportion of cells expressing Foxp3.

Previous data showed that tolerance induced with Ac1-9(4Tyr) was observed only if assessed 4 days after peptide administration (Liu and Wraith, 1995). However, studies that investigated the induction or expansion of Tregs which resemble naturally arising Tregs have described emergence of these only after 10 days (Apostolou and von Boehmer, 2004) and routinely assess Treg phenotype and function up to two weeks after treatment (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). We therefore assessed the capacity of 100μg Ac(1-9)4Tyr treatment to expand Foxp3+ T cells 14 days after antigen administration (Fig. 4.4). The proportion of Tg4 T cells in Ac1-9(4Tyr) treated mice was not significantly higher than in PBS treated mice at this time (compare Fig. 4.4A and B). The proportion of Foxp3+ cells within the Tg4 populations was also similar in both PBS and Ac1-9(4Tyr) treated mice (Fig. 4.4A and B). However, cells from 4Tyr treated mice were completely unresponsive to in vitro restimulation with

MBP(Ac1-9), as assessed by 3H-thymidine incorporation (Fig. 4.4B). This suggested that 6 days after i.v. administration of Ac1-9(4Tyr) the transferred population of Tg4 cells were tolerant to re-stimulation in vitro. After 14 days, the proportion of Tg4 cells in mice treated with Ac1-9(4Tyr) had contracted to similar levels in PBS-treated mice The cells from Ac1-9(4Tyr) treated mice maintained the levels of unresponsiveness seen at the earlier timepoints. Ac1-9(4Tyr) treatment did not significantly increase the proportions of Tg4 Foxp3+ cells at any timepoint studied.

4.2.2.2 Ac1-9(4Tyr) treatment suppressed disease induced with WT MBP (Ac1-9)

To assess the capacity of Ac1-9(4Tyr) to induce tolerance in response to MBP in vivo, CD4+ Tg4 cells were adoptively transferred to B10.PLxC57BL/6 hosts (H-2u x H-2s). Recipients were treated with 200μg Ac1-9(4Tyr) then immunised with MBP(Ac1-9) 7 days later to assess EAE (Fig. 4.5A). Previous investigations from our group have shown that B10.PLxC57BL/6 mice require transfer of Tg4 T cells to develop EAE after immunisation with MBP (McCue et al., 2004), therefore any effect of Ac1-9(4Tyr) administration on EAE should be due to the effect of the peptide on the transferred Tg4 population. As shown in Fig. 4.5B, mice that had been treated with a single dose of 4Tyr one day after the transfer of Tg4 cells were protected from EAE induced with MBP(Ac1-9). Mice that received Tg4 cells, and received either WT MBP(Ac1-9) or PBS were not protected from disease. These data demonstrated that treatment with Ac1-9(4Tyr) had effectively tolerised the Tg4 cells that had previously been transferred. This was sufficient to prevent the induction of EAE. Tg4 cells could be detected in the CNS of both 4Tyr and PBS treated mice and showed an approximate two fold increase in the proportions of Tg4 cells expressing Foxp3, from ~20% Tg4 Foxp3+ cells in PBS treated to ~40% Tg4 Foxp3+ cells in Ac1-9(4Tyr) treated mice (Joanne Konkel, unpublished observations). This enhanced proportion of Foxp3-expressing Tg4 cells was only observed in the CNS Ac1-9(4Tyr) treated mice during EAE, suggesting that the CNS

inflammatory environment may be required to drive the expression or upregulation of Foxp3. Mice that were treated with Ac1-9(4Tyr) but were not induced to develop EAE did not show changes in the proportions of Foxp3+ cells in the transferred Tg4 population. It is not clear if the Tg4 Foxp3+ cells seen in the CNS are expanded from the 2-3% Foxp3+ cells from the initiating population or Foxp3- cells that have up-regulated the expression of Foxp3+. This could be assessed by sorting Foxp3-GFP negative populations prior to cell transfer.

4.2.3 Can antigen-specific Tregs suppress disease induced with another peptide?

To address the capacity of the in vitro expanded MBP-reactive Tregs to suppress disease induced against distinct myelin antigens, a different EAE model and strain of mouse was used. B10.PLxSJL mice express both H-2^u and H-2^s and can be induced to develop EAE by immunisation with spinal cord homogenate (SCH), MBP and PLP (Miller et al., 1995). Immunisation with one peptide (e.g. Ac1-9) can initiate an I-A^u restricted response, but the response can then spread to I-E^u and I-A^s restricted epitopes. By using this model we can assess the ability of Tregs reactive to one epitope to suppress disease induced with an epitope from another distinct CNS protein. To do this, we sorted CD4+CD25+CD62L^{hi} Tregs from Tg4 mice and expanded them in vitro. After 7-10 days $3x10^5$ Tregs were transferred i.v. to B10.PLxSJL mice. Mice were immunised with either MBP(Ac1-9) or PLP(139-151) one day after Treg transfer and assessed for clinical signs of EAE over the next 60 days (Fig. 4.6A). Mice that were immunised with MBP and received no Tregs developed a severe relapsing course of disease, where one out of four mice died as a result of disease severity (Fig. 4.6B). Mice that had received expanded Tg4 Tregs prior to MBP-immunisation were completely protected from the first episode of disease (Fig. 4.6B). At day 37 post-immunisation, 1 out of 4 mice developed clinical signs of EAE and rapidly progressed to death (Fig 4.6B). All other

mice that received Tg4 Tregs remained free of disease until the end of the 60 day observation period.

Mice that were immunised with PLP(139-151) and had not received Tregs also underwent a relapsing course of disease and the onset of disease was not affected in mice which had received Tg4 Tregs one day prior to PLP-immunisation (Fig 4.6B). However, the mice treated with Tg4 Tregs began their recovery at a much earlier time-point than the PBS treated group and also showed reduced incidence of relapses over the 60 day period. The disease observed from day 25 to day 60 in the group that received Tg4 Tregs is due to chronic disease of one mouse in this group that remained at grade 2-3 throughout the 60 days, while 75% of mice in the PBS treated group underwent relapses at some stage over this period.

These data suggest that MBP-reactive Tregs can suppress disease induced with another antigen. This has important implications for Treg based therapies as it may be possible to target one antigen, but also suppress effector T cells reactive to other antigens in the vicinity.

4.2.3.1 In vivo peptide therapy to suppress disease induced against multiple epitopes

We next attempted to utilise the Ac1-9(4Tyr) APL to prevent disease in the B10.PLxSJL model and to assess if peptide-induced tolerance against MBP(Ac1-9) could prevent EAE initiated against PLP(139-151). To confirm that Ac1-9(4Tyr)-treatment suppressed MBP-induced EAE in the B10.PLxSJL model, we transferred $2x10^6$ CD4-purified Tg4 cells i.v. followed by Ac1-9(4Tyr) i.v. one day later. Mice were then immunised seven days later with MBP(Ac1-9) (Fig. 4.7A) or PLP(139-151) (Fig. 4.8A). Mice that had been immunised with MBP(Ac1-9) and pre-treated with Ac1-9(4Tyr) showed a dramatic reduction in disease incidence and severity compared to mice which received PBS (Fig.

4.7A). Two mice per group were harvested at day 7 of EAE and assessed for the presence of Tg4 cells in the CNS, lymph node and spleen via FACS by the absence of Ly5.1 on the surface of the transferred Tg4 cells (Fig. 4.7B). PBS treated mice had a greatly enhanced proportion of Tg4 cells in the CNS compared to Ac1-9(4Tyr) treated mice. When the cells from the CNS and LN were re-stimulated with MBP in vitro, Tg4 cells from PBS treated mice showed a higher level of IFNγ and IL-17 production compared to the Tg4 cells from Ac1-9(4Tyr) treated mice (Fig. 4.7C and D), suggesting that the peptide treatment had tolerised the transferred Tg4 cells in vivo. Peptide treatment also reduced the levels of IFNγ and IL-17 produced by host CD4+ T cells in the CNS and LN in response to MBP (data not shown). The Tg4 cells from Ac1-9(4Tyr)-treated mice in this system did not express Foxp3 (data not shown) but did show reduced levels of effector cytokine production. The severity of disease in PBS treated mice could be attributed to the high levels of Tg4 cells in the CNS with the capacity to produce IFNγ and IL-17.

In mice immunised with PLP, Ac1-9(4Tyr) treatment did not affect disease development or the severity of primary disease, however, mice which had received Ac1-9(4Tyr) were completely protected from relapses (Fig. 4.8B). 3 mice per group were harvested at day 14 and the proportion of Tg4 cells and Foxp3-expression assessed. Similar to the MBP-immunised mice, PBS-treated mice showed a much higher proportion of Tg4 cells in the CNS (Fig. 4.8C). No Foxp3 could be detected in the Tg4 cells of either PBS or 4Tyr treated mice (data not shown) while the proportion of Foxp3+ cells in the CNS of host CD4+ cells was not significantly different (Fig. 4.8D).

Lymphocytes from the CNS were assessed for cytokine production upon re-stimulation with WT MBP(Ac1-9) or PLP(139-151) in vitro. No cytokine production or Foxp3 expression could be detected in the Tg4 cells in this experiment (data not shown). Host CD4+ cells from the CNS of PBS-treated mice consistently produced higher levels of IFNγ and IL-17 when stimulated with MBP(Ac1-9) and PLP(139-151) than Ac1-9(4Tyr)-treated mice (Fig. 4.9A). However, the levels of IL-10 produced by total CD4+

cells from the CNS of Ac1-9(4Tyr)-treated mice was higher than in cells from PBS-treated mice (Fig. 4.9B). These data suggest that in the recovery stage of primary disease induced with PLP(139-151), CD4+ T cells in the CNS of mice which had been transferred with Tg4 cells and treated with Ac1-9(4Tyr) produced high levels of IL-10 and lower levels of effector cytokines in response to myelin antigens compared to PBS treated mice. This may contribute to a regulatory environment which prevented relapses in Ac1-9(4Tyr) treated mice, however the levels of Foxp3 were not enhanced in 4Tyr treated vs. PBS treated mice suggesting that the mechanism of regulation is not via expansion or induction of Foxp3+ Tregs.

4.2.4 Does the inflamed CNS influence the balance between Foxp3+ and Foxp3- Tg4 cells?

It had previously been shown that the transfer of CD4+Tg4 cells prior to immunisation with MBP(Ac1-9) or with PLP(139-151) resulted in the appearance of Tg4 cells in the CNS and of which up to 20% expressed Foxp3 (Steve Anderton, personal communication). This was not seen when mice were immunised with either peptide, but not induced to develop EAE (i.e. no pertussis toxin administered). This led us to question wether the inflamed CNS could drive the expansion of Foxp3+ cells resulting in their enhanced frequency. Furthermore, data from this laboratory had indicated that administration of Ac1-9(4Tyr) to hosts that had received Tg4 cells could further enhance the proportion of Tg4 Foxp3+ cells in the CNS to 40%. This suggested that prior exposure to Ac1-9(4Tyr) could influence the capacity of the CNS to drive the expansion of Foxp3+ cells in situ.

To test this hypothesis, CD4+ cells were sorted from Tg4 mice that had been treated with 200μg Ac1-9(4Tyr) or PBS 6 days earlier. $2x10^6$ cells were then transferred i.v. to B10.PLxSJL hosts that had been immunised with MBP(Ac1-9) 10 days earlier and had developed clinical signs of EAE (Fig. 4.10A). Mice were left for 7 days after the Tg4

cell transfer, then CNS, LN and spleen were harvested at day 17 post EAE induction (Fig. 4.10B). The proportion of Tg4 cells in each organ and the frequency of Foxp3+ cells within the Tg4 populations were assessed by FACS (Fig. 4.10B and C). Mice that had received Ac1-9(4Tyr)-treated Tg4 cells did not show any significant difference in disease course initially compared to mice which had received PBS treated Tg4 cells or to un-transferred mice, however at later stages the mice receiving 4Tyr-treated cells appeared to have a prolonged disease compared to the other groups (Fig. 4.10B). The proportion of Tg4 cells in mice that had been given Ac1-9(4Tyr)-treated Tg4 cells was not significantly greater than PBS treated Tg4 cells in the CNS, spleen or LN (Fig. 4.10C). Also, the proportion of Tg4 Foxp3+ cells in the CNS and LN was not significantly different in Ac1-9(4Tyr) vs. PBS pre-treated groups. Although the Ac1-9(4Tyr) treated Tg4 cells showed a reduction in the proportion of Foxp3+ cells in the spleen, this could only be assessed in one animal as the others had died as a result of EAE severity (Fig. 4.10D).

To assess the persistence of Tg4 cells in PLP(139-151) immunised mice, the experiment was repeated using PLP(139-151) immunisation instead of MBP(Ac1-9) (Fig. 4.11A). In this setting, the mice that received Ac1-9(4Tyr)-treated Tg4 cells did not show enhanced disease compared to non-transferred mice (Fig. 4.11B). However, mice that received PBS-treated Tg4 cells showed a prolonged and more severe disease course (Fig. 4.11B). No significant difference in the proportions of Tg4 cells in any organ could be detected (Fig. 4.11C). Similarly, there was no significant difference in the proportions of Foxp3+ Tg4 cells in the spleen, LN or CNS; however the Ac1-9(4Tyr)-treated Tg4 cells in both CNS and LN showed a trend towards reduced proportions of Foxp3+ cells compared to PBS-treated Tg4 cells (Fig. 4.11D).

When splenocytes from each group were assessed for proliferation to PLP(139-151) in vitro, no significant difference was observed between the three groups (Fig. 4.12A). When stimulated with MBP(Ac1-9), both of the Tg4 transferred groups showed higher levels of proliferation compared to un-transferred mice, showing that both the PBS and

Ac1-9(4Tyr) treated Tg4 cells were capable of responding to MBP(Ac1-9) to a similar level in vitro (Fig. 4.12B). Cells from the CNS of each mouse were assessed for cytokine production in vitro. In response to MBP(Ac1-9) stimulation a higher proportion of the Ac1-9(4Tyr)-treated Tg4 cells from the CNS of PLP(139-151) immunised mice produced IFNγ and IL-17 compared to PBS treated mice (Fig. 4.12C). While a proportion of 4Tyr treated Tg4 cells from the CNS of PLP(139-151) immunised mice also produced IFNγ in response to PLP(139-151) stimulation in vitro (Fig. 4.12D), this was not significantly higher than the background levels of proliferation.

4.3 Discussion

It has been documented that while the number of Tregs in MS patients are in the normal range, Tregs isolated from the patients are defective in their capacity to suppress effector T cells (Haas et al., 2005; Venken et al., 2008b; Viglietta et al., 2004). Developing protocols to expand Tregs and enhance their function through Treg based therapies is therefore of great interest. Here we have shown that purification and expansion of CD4+CD25+CD62L^{hi} Tg4 Tregs results in a population of MBP(Ac1-9)-reactive Tregs that maintains expression of Foxp3 and can suppress disease induced with the same or distinct antigen. We further attempted to expand Foxp3+ Tregs in vivo using peptide based therapy. We found that protection from EAE could be achieved using Ac1-9(4Tyr), an APL with high affinity for MHC, that maintained a population of peptide-reactive cells although the proportion of these cells that expressed Foxp3 was not enhanced. Importantly these data also show that tolerance induced against MBP(Ac1-9) resulted in reduced disease relapses when induced with PLP(139-151), a distinct myelin antigen.

Defective Treg function is not limited to MS, but also noted in studies of patients with rheumatoid arthritis (Ehrenstein et al., 2004) and type 1 diabetes (Lindley et al., 2005) therefore developing protocols to generate sufficient Treg numbers for adoptive transfer therapy would be beneficial in a number of disease settings. A major issue in both mouse and human studies is that Tregs represent a small population of CD4+ T cells (2-10%), therefore isolation of enough cell numbers to transfer therapeutically is difficult. Isolation and expansion of Tregs in vivo represents one mechanism whereby the numbers of Tregs can be enhanced. Initially, it was reported that Tregs were anergic to antigenic stimulation in vitro (Jonuleit et al., 2001; Walker et al., 2003). In vivo, Tregs require stimulation via the TCR and CD28 co-stimulation for their homeostasis (Salomon et al., 2000; Tang et al., 2003). They are also critically dependent on IL-2 for their expansion in vivo (Bayer et al., 2005; Setoguchi et al., 2005). It is therefore

unsurprising that these three components (TCR stimulation, CD28 co-stimulation and IL-2) have now been shown to efficiently expand both murine and human Tregs in vitro (Chai et al., 2008; Dieckmann et al., 2001; Earle et al., 2005; Fisson et al., 2006; Hoffmann et al., 2004; Nishimura et al., 2004).

In our studies, we have shown that antigen-specific Tregs are more potent at suppressing disease than polyclonal Tregs (Fig. 4.2). The increased potential of disease relevant Tregs to suppress inflammation has also been demonstrated in other models (Masteller et al., 2005; Tang et al., 2004b; Tarbell et al., 2004). It is important to note that the frequency of antigen specific Tregs in a polyclonal population is obviously far reduced compared to the expansion of antigen-reactive Tregs by the expansion of TCR transgenic T cells or after antigen-specific selection from a polyclonal repertoire, as can be achieved using peptide:MHC instead of anti-CD3/anti-CD28 stimulation (Fisson et al., 2006). Tarbell et al. (Tarbell et al., 2004) used islet antigen specific Tregs from BDC2.5 TCR transgenic mice that were expanded in vitro using DCs from NOD mice and showed that transfer of only 5000 islet-specific Tregs could give disease suppression. In a different model, Taylor et al. showed that 1-2x10^6 polyclonal in vitro expanded Tregs were required to prevent graft versus host disease (GVHD) (Taylor et al., 2002). This suggests that the specific targeting of antigen-reactive Tregs will be more effective for therapy as fewer cells are required for suppression and are more effective than polyclonal Tregs.

The use of transgenic systems allows antigen reactive cells to be tracked and assessed ex vivo. However, the ability to convert studies using transgenic models to polyclonal populations will be difficult, as has been highlighted by the Bluestone group (Masteller et al., 2005). Using IL-2 and beads coated with recombinant islet peptide-mimic:MHC and anti-CD28 mAb, their study investigated the capacity of expanded islet reactive Tregs to suppress diabetes in NOD mice. By mixing transgenic Tregs at various ratios with polyclonal Tregs it was shown that the antigen coated beads preferentially expanded the antigen-reactive Tregs in vitro from a frequency as low as 1:100,000. They

also showed that islet-reactive Tregs could be expanded from polyclonal populations, but that these Tregs were not as effective as the transgenic expanded Tregs in suppressing diabetes. It was noted that expanded transgenic Treg cultures had higher levels of IL-10 and TGFβ compared to polyclonal expanded cultures and that while WT expanded Tregs were oligoclonal, as assessed by TCR expression, they showed varying avidities for p:MHC class II multimers. These data highlight that while transgenic systems are useful to track antigen-reactive cells in vivo, care must be taken when investigating the potential of Treg therapy in non-transgenic populations.

Other considerations must be made with regard to the ex vivo expansion of Tregs. Technical issues include the source of T cells from which to expand Treg populations. Human T cell isolation is largely restricted to peripheral blood as it is a relatively non-invasive procedure. However, the most potent and disease relevant Tregs will likely be focused at the site of inflammation, which for MS presents an obvious problem. The ability to expand myelin-reactive T cells from the peripheral blood of MS patients has been described (Arbour et al., 2006) and could potentially be tailored towards the expansion of myelin reactive regulatory cells. Another major consideration is that while the findings of many mouse studies have been replicated in human studies, there are a number of differences in mouse and human Tregs. For example, while mouse Tregs lack expression of MHC class II, human Tregs are MHC class II bright as assessed by flow cytometry (June and Blazar, 2006) and these differences may affect the capacity to use human Tregs in a similar way to mouse Tregs.

The main issue in our expansion protocol was the apparent loss of Foxp3-expression from Treg cultures after the 7 day in vitro expansion step. Although most of the Treg populations had initially been sorted to a high purity of Foxp3+ cells, the frequency of Foxp3-expressing cells was reduced to varying degrees after expansion (Fig. 4.1B). It could be argued that the reduced frequency of Foxp3-expressing cells was solely due to the selective out-growth of Foxp3 negative cells, however, this population represented a very small fraction of the sorted population and would seem an unlikely explanation.

The death of Foxp3+ cells could also have contributed to the loss of Foxp3+ cells in culture. However this scenario would also seem unlikely as we would have expected fewer cells to be recovered from the cultures at day 7 and this was not the case. It has also been suggested that Tregs show an intrinsic resistance to TCR-signalling induced apoptosis relative to conventional T cells, which also argues against the deletion of Foxp3+ cells (Taylor et al., 2007). The levels of cell death in culture could be assessed using Annexin V staining.

The remaining scenario to explain the reduced proportions of Foxp3+ cell after expansion is the selective loss of Foxp3-expression by some of the cells in culture. The lineage decisions made by a developing Treg have been investigated extensively over the last few years, as discussed in Chapter 1. Of particular interest is wether Foxp3-expression and Treg status is fixed or if cells within this lineage demonstrate some degree of plasticity. The stability of Foxp3 expression has more recently been accredited to the epigenetic modulations at the foxp3 locus. Comparisons of CD25- and CD25+ cells showed differences in the methylation status of the foxp3 locus, such that in CD25+ cells the CpG motifs on the foxp3 locus were almost completely demethylated in one region, while they were methylated in the CD25- population (Floess et al., 2007). They also showed that developing Treg thymocytes show a partial degree of de-methylation at the foxp3 locus, while full de-methylation is only evident on mature Tregs which are about to exit the thymus. In contrast to this finding in naturally occurring Tregs, the foxp3 locus of Tregs induced by culture with TGFβ showed reduced de-methylation and this was rapidly lost upon further stimulation without additional TGFβ. These results suggest that only cells which are committed to the Treg lineage, i.e. originating in the thymus, will stably express Foxp3 due the epigenetic imprinting given to these cells upon development.

However, the targeting of antigen to DC in the steady state via DEC-205 has shown to generate Foxp3+ Tregs which maintain Foxp3 expression for up to 9 weeks even in the absence of cognate antigen (Kretschmer et al., 2005). This has recently been shown to be

due to the change in DNA methylation, such that Tregs by this protocol showed similar de-methylation in the foxp3 locus as that of naturally occurring Tregs (Polansky et al., 2008). This paper showed strong evidence to suggest that there is a functional link between DNA de-methylation of the Treg-specific de-methylation region (TSDR) on the foxp3 locus and stable Foxp3 expression. Interestingly, Foxp3-expression has been shown to be up-regulated at some stage in all activated human T cells, merely a product of T cell activation designed to dampen down the response (Pillai and Karandikar, 2007). This would make sense to dampen T cell responses late in the response to prevent excessive pathology and tissue damage. Foxp3 expression in most of these cells was shown to be rapidly lost, with only a few cells maintaining expression. This residual Treg population would enhance the Treg repertoire by adding to the TCR diversity in these cells.

Collectively these results suggest that Foxp3 expression may be up-regulated in some cells at inflamed sites but that this expression is not stable. These studies have important implications in Treg-based therapies. It would be highly dangerous to expand Tregs to an auto-antigen in the hope of reducing autoimmunity if these cells would have the capacity to lose regulatory activity and convert to an auto-aggressive effector T cell. Indeed, this has been documented recently with the discovery of the IL-17 producing CD4+ T cell population, Th17 cells (Langrish et al., 2005). The pathway which leads to differentiation of Th17+ cells overlaps with that of Tregs as both populations utilise TGFβ. In the presence of TGFβ, cells are directed towards, a Treg/Foxp3+ fate. However, addition of IL-6 to the culture can drive a pathogenic Th17/RORγT+ population, proposed to be instrumental in many pathologies which were classically associated with Th1 cells (Bettelli et al., 2006b). This would then suggest that one cell could become either a regulatory cell or pathogenic cell depending on the cytokine milieu (Weaver et al., 2006; Xu et al., 2007) and that Tregs themselves can be converted to Th17+ cells (Xu et al., 2007). If committed Tregs which have fully demthylated TSDR can also be driven towards a Th17 fate is as yet unknown.

Ultimately, to overcome many of the problems and potential dangers of ex vivo Tregs expansion, direct expansion of antigen-reactive Tregs in vivo is desirable. We have used an APL of MBP(Ac1-9) with an amino acid substitution at position 4 from Lys to Tyr (4Tyr) that binds with a higher affinity to I-Au (Fairchild et al., 1993). Previous studies using this APL showed that treatment with 100ug Ac1-9(4Tyr) i.p. either before or after the induction of EAE with MBP(Ac1-9) protected or reduced disease respectively (Liu and Wraith, 1995). As Ac1-9(4Tyr) binds to MHC with high affinity, it could be suggested that the peptide functioned by blocking the MHC, however this was ruled out as a single dose of 4Tyr given after disease induction was sufficient to reduce disease. This study also ruled out class switching from Th1 to Th2 as reductions in the levels of both IFNγ and IL-4 were observed. Cells from 4Tyr treated mice did not suppress cells from Ac1-9-primed mice, suggesting that the effect was not mediated by regulatory T cells; however these observations were made before definitive markers for Tregs were identified.

Later studies determined that a single intranasal high dose (100ug) of Ac1-9(4Tyr) resulted in priming of T cells in Tg4 TCR transgenic mice, while multiple doses resulted in tolerance (Burkhart et al., 1999; Sundstedt et al., 2003). Both studies demonstrated a role for IL-10 in suppression. The tolerant cells generated by Sundstedt et al. (Sundstedt et al., 2003) were mostly CD25-CTLA-4+ and anergic to antigenic stimulation in vitro; however, the anergic state could be reversed by the addition of IL-2. Burkhart et al. however report that antigen-induced unresponsiveness could not be reversed by the addition of IL-2 (Burkhart et al., 1999). The experiments described in this chapter showed that treatment with Ac1-9(4Tyr) results in expansion of adoptively transferred peptide-reactive T cells and tolerance to peptide stimulation but did not increase the proportion of cells that express Foxp3 (Fig. 4.2). Peptide treatment reduced the proportion of MBP(Ac1-9) reactive T cells in the CNS, suppressed MBP(Ac1-9)-induced EAE (Fig. 4.7) and reduced relapses in PLP(139-151)-induced EAE (Fig. 4.8). IL-10 production could not be detected by the TCR transgenic cells in any experiments using i.v. administration of Ac1-9(4Tyr) although this could be due to restriction in the

sensitivity of the assays used to detect IL-10 in these studies. The differences observed in this study and those of Burkhart et al. and Sundstedt et al. could be reflective of the differences in route of administration (i.v. vs. i.n.) or antigen dose (1 x 200μg vs. 10 x 100μg), however we similarly did not detect the expression of Foxp3 in peptide treated cells. Interestingly, while some studies have shown deletion of most peptide reactive T cells after antigen administration (Critchfield et al., 1994; Liblau et al., 1996) a more recent study has shown that anergy is sufficient to induce tolerance in T cells that are deficient in the pro-apoptotic molecule, Bim (and therefore cannot undergo deletion) (Barron et al., 2008). It would be of interest to assess the levels of apoptotic and anergic markers in Tg4 cells to gain a greater understanding of the signals imprinted after Ac1-9(4Tyr) encounter.

It has been reported that i.v. administration of antigen in the absence of inflammation results in the expansion of Foxp3+ Tregs while Foxp3- T cells undergo abortive activation after interaction with CD11c+ DC in the spleen (Chappert et al., 2008). The expansion of Foxp3+ T cells was not evident in the 4Tyr treated groups in our Tg4 transfer model (Fig. 4.3). Other studies that have documented the expansion or generation of Foxp3+ T cells have used low dose antigen under non-inflammatory conditions by targeting antigen to immature DCs (Kretschmer et al., 2005) or by subcutaneous peptide infusion using osmotic mini-pumps (Apostolou and von Boehmer, 2004; Verginis et al., 2008). The conversion of Foxp3- T cells to Foxp3+ was observed only under sub-immunogenic conditions where T cells did not undergo extensive proliferation. While T cells that proliferated vigorously initially upregulated Foxp3, this was lost over time suggesting that upregulation of Foxp3 is only transient under strong antigenic stimuli (Kretschmer et al., 2005). Stimulation with 200μg Ac1-9(4Tyr) has been shown to induce strong proliferation of Tg4 cells in vivo (Joanne Konkel, unpublished observations). High dose antigen administration and proliferation has been reported to favour the induction of Tr1 cells (Battaglia et al., 2006a). Thus, Ac1-9(4Tyr) stimulation may be unfavourable for the induction or expansion of Foxp3+ Tregs and this could explain why we find no increase in the frequency of Ac1-9-reactive Foxp3+

cells when this peptide is administered at high doses intravenously. The exact mechanism underlying 4Tyr induced tolerance remains to be clarified.

B10.PLxSJL mice that had received Tg4 Tregs prior to disease induction showed fewer relapses after immunization with PLP(139-151) (Fig. 4.6B). As Ac1-9(4Tyr) has been shown to persist for longer periods in vivo than WT MBP(Ac1-9) it may be suggested that Ac1-9(4Tyr) functions to maintain the transferred Tg4 cells until CNS destruction occurs and releases epitopes of MBP that could activate Tg4 Tregs. In this scenario, MBP-reactive Tregs require encounter with their cognate antigen to become activated to suppress. Once the Tg4 Tregs gain access to the CNS they may encounter MBP(Ac1-9) and become activated to control the inflammation in situ. Other work in the lab has demonstrated that co-immunisation with PLP(139-151) and MBP(Ac1-9) further enhanced the suppressive capacity of Tg4 Tregs (Leigh Stephens, unpublished data), suggesting that encounter with cognate antigen further potentiates the ability of Tg4 Tregs to suppress disease targeted against distinct myelin antigens.

To determine if encounter with Ac1-9(4Tyr) is sufficient to imprint regulatory function we treated Tg4 mice directly with Ac1-9(4Tyr). After 6 days, CD4+ cells were sorted from the Tg4 mice and transferred to mice which had been immunised with MBP or PLP 10 days earlier (Fig. 4.11A). After 7 days Tg4 cells in the mice were assessed for Foxp3 expression and cytokine production to determine if the proportion of Foxp3+ cells was higher in cells that had previously encountered Ac1-9(4Tyr). It was found that both PBS and Ac1-9(4Tyr) pre-treated Tg4 cells infiltrated the CNS and but that Ac1-9(4Tyr) pre-treated cells produced greater amounts of IFNγ and IL-17 after peptide stimulation in vitro (Fig. 4.11 and 4.12). Single doses of 100μg Ac1-9(4Tyr) have been shown to induce hyper-responsiveness to MBP(Ac1-9) stimulation when given to MBP-reactive transgenic mice, however tolerance can be induced after multiple doses of Ac1-9(4Tyr) are administered (Burkhart et al., 1999; Liu et al., 1995). It would be important to repeat this experiment using a protocol that will induce tolerance in the Tg4 mice prior to cell

recovery and transfer, as administration of a single shot of 200μg Ac1-9(4Tyr) will have been unlikely to induce tolerance in this setting, although this was not investigated here.

While these studies using peptide based therapies have shown protective effects in mouse models, there exist a number of questions regarding the feasibility of these systems in human disease. Firstly, the use of peptide administration (WT and APLs) as therapy will be restricted in human disease where the antigens are poorly defined. Peptides that have been altered in the key TCR contact residues have been shown to alter T cell responses from the generation of peptides with full agonistic properties to complete antagonism (Evavold et al., 1993). APLs that antagonise TCR binding have been shown to be effective in the suppression of EAE (Franco et al., 1994; Kuchroo et al., 1994; Nicholson et al., 1997). However, care must be taken as data show that while an APL may act as an antagonist for one T cell, the effect could be markedly different in another (Anderton et al., 1998). The dangers of this were highlighted in clinical trials using an MBP(83-99) APL in MS patients where disease was exacerbated by APL-induced expansion and activation of MBP-reactive encephalitogenic T cells (Bielekova et al., 2000). Secondly, it has been suggested that selective peptide based therapy will be of limited efficacy in chronic human diseases that show a diverse autoimmune repertoire (Lehmann et al., 1993; McCarron et al., 1990). However, during chronic responses, T cells will receive continual antigenic stimulation. Repeated antigenic stimulation has been shown to drive the preferential selection of certain clonotypes within polyclonal populations in vitro and antigen based repertoire restriction may also occur in MS as T cells from chronic plaques have shown restricted T cell repertoires (Wucherpfennig et al., 1992). Furthermore, recent studies have reported that antigen-reactive Tregs also show restricted repertoires during EAE (Madakamutil et al., 2008). These findings suggest that while the immune response in MS is diverse, a degree of repertoire restriction occurs and that these cells may be preferentially targeted in vivo.

Taken together, the data presented here demonstrated that in vitro expansion and transfer of Foxp3+ Tregs that are reactive to one myelin antigen can suppress disease induced

with the same, but also distinct, myelin antigens. This can be extended to peptide based tolerance, whereby tolerance induced against one antigen can also affect the immune response to distinct antigens. These data have important implications in antigen based therapies, particularly for inflammatory conditions that will involve immune responses targeted against a number of different antigens over the course of disease. While these studies have not identified expansion of Foxp3+ Tregs using peptide in vivo, the questions of Foxp3 stability in expanded and induced Tregs highlight that the development of protocols to expand Foxp3+ cells will require long term tracking of Foxp3-expression in these cells. By determining the antigenic reactivity of Tregs in the CNS it is hoped that mechanisms to target the preferential expansion of pre-committed, disease relevant T cells that stably express Foxp3+ will provide potential antigen-based therapeutic targets to prevent and treat CNS inflammation.

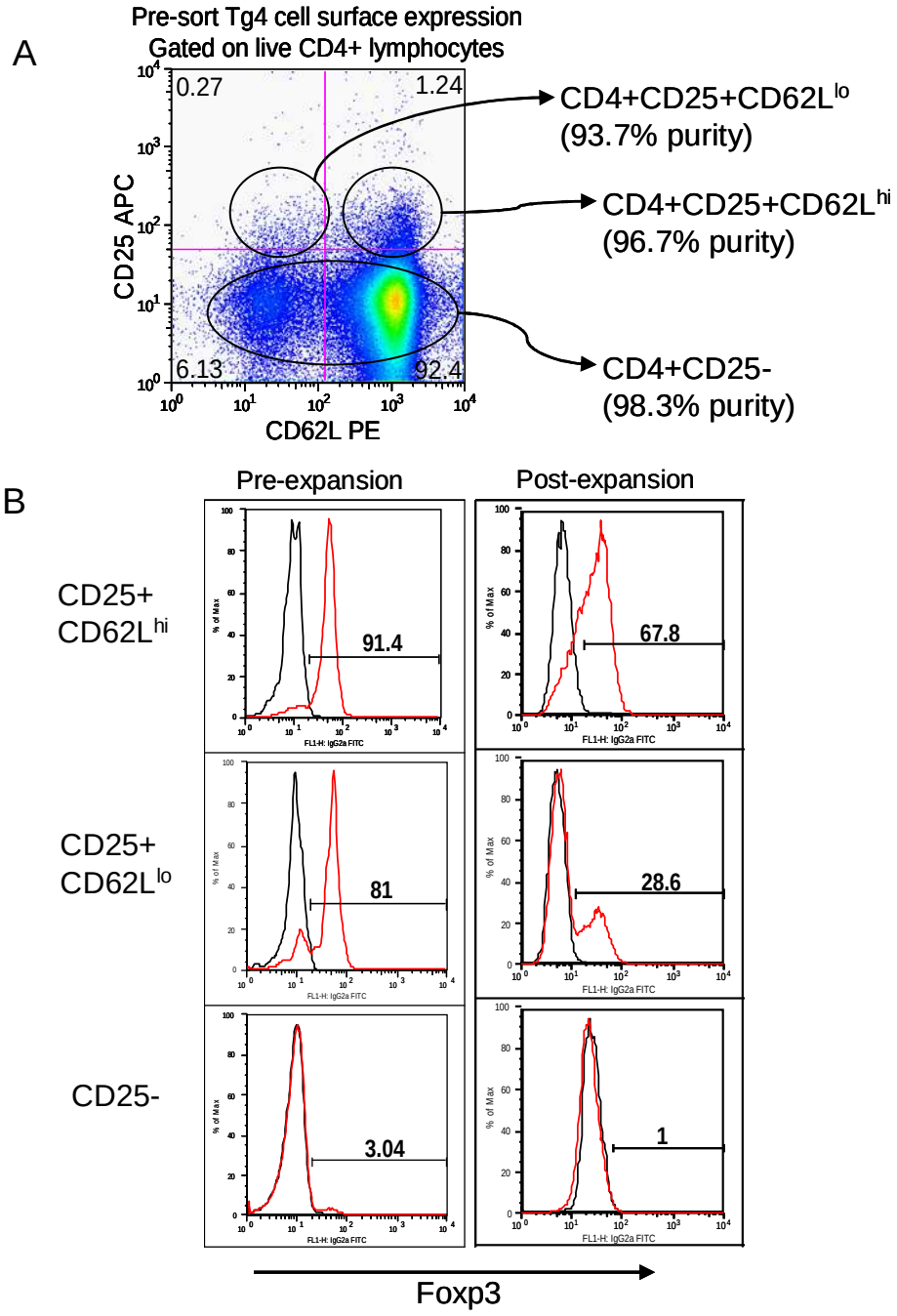


Figure 4.1: Tg4 CD25+CD62L^{hi} sorted cells maintain Foxp3 expression after expansion. A: Representative plot of Tg4 cell surface expression on whole CD4+ purified splenocytes pre-sort. Each group was sorted to high purity on FACSAria under sterile conditions. Cells were then expanded in RPMI-5% FCS plus anti-CD3/anti-CD28 coated beads + 1000U/ml IL-2 for 7 days. B: Foxp3 expression by each subset before (left hand column) and after (right hand column) in vitro expansion. Black line = isotype control, red line = Foxp3 staining. Data represents one of four separate experiments with similar results.

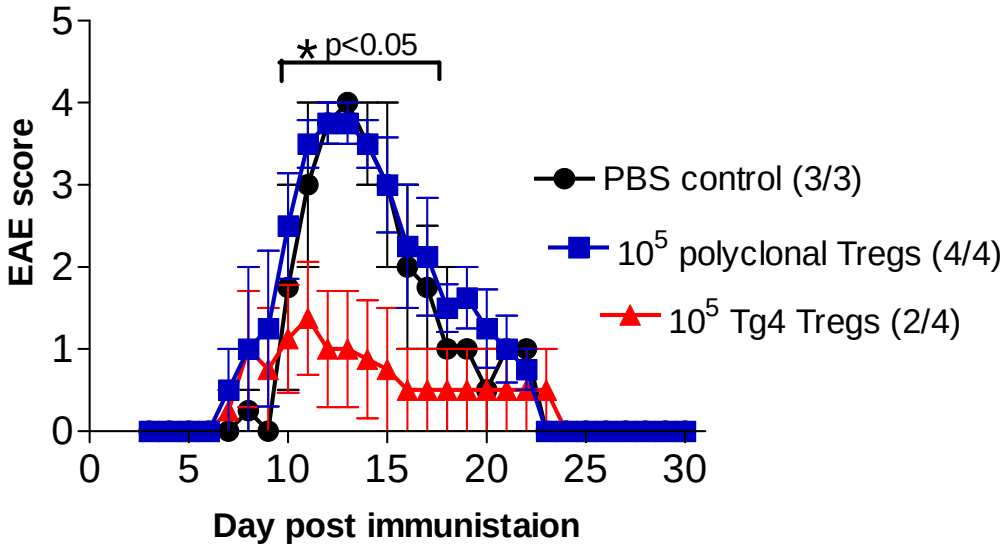


Figure 4.2: In vitro expanded CD4+CD25+CD62L^{hi} cells from naïve Tg4 transgenic mice suppress MBP(Ac1-9) induced EAE better than polyclonal Tregs. B10.PL mice received 1.5x10^5 CD4+CD25+CD62L^{hi} cells sorted from Tg4 mice (Δ) or B10.PL mice (□) or no transfer (○) one day prior to EAE induction with 100µg MBP(Ac1-9)/CFA. Numbers in brackets represents incidence of disease. Data is representative of two individual experiments with 3-4 mice per group. * = p<0.05. Statistical difference between day 10-17 of EAE assessed by Mann Whitney of cumulative disease scores per group, per day.

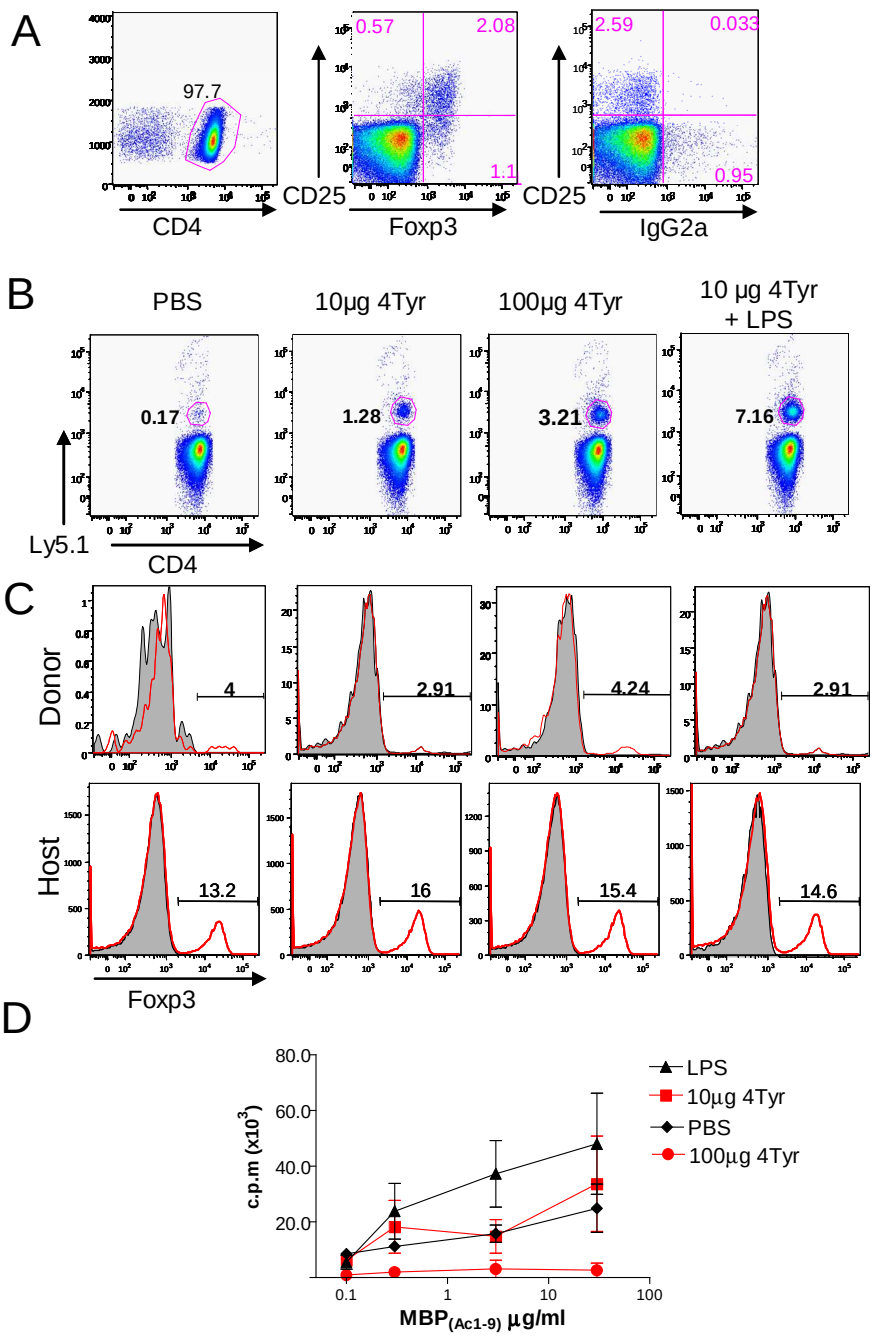


Figure 4.3: Treatment with high dose Ac1-9(4Tyr) expands MBP-reactive cells in vivo and tolerises the response to WT MBP in vitro. A: Tg4Ly5.1+ cells were purified by MACS to ~98% CD4+. Cells were assessed for Foxp3 expression prior to i.v. transfer to B10.PL hosts ($2x10^6$/mouse). 1 day post cell transfer mice received PBS, 10µg or 100µg 4Tyr or 10µg 4Tyr plus 20µg LPS i.v. B: 6 days later spleens were harvested and the proportion of Tg4 cells assessed by FACS. C: Proportion of Foxp3+ cells in Tg4 cell (top panels) and host cells (bottom panels). Filled plots = isotype control. All plots show one mouse representative of three mice per group D: Splenocyte proliferation in response to MBP(Ac1-9) in vitro assessed by ^{3}H-thymidine incorporation, error bars showing 3 individual mice per group. Data representative of two independent experiments with simlar results.

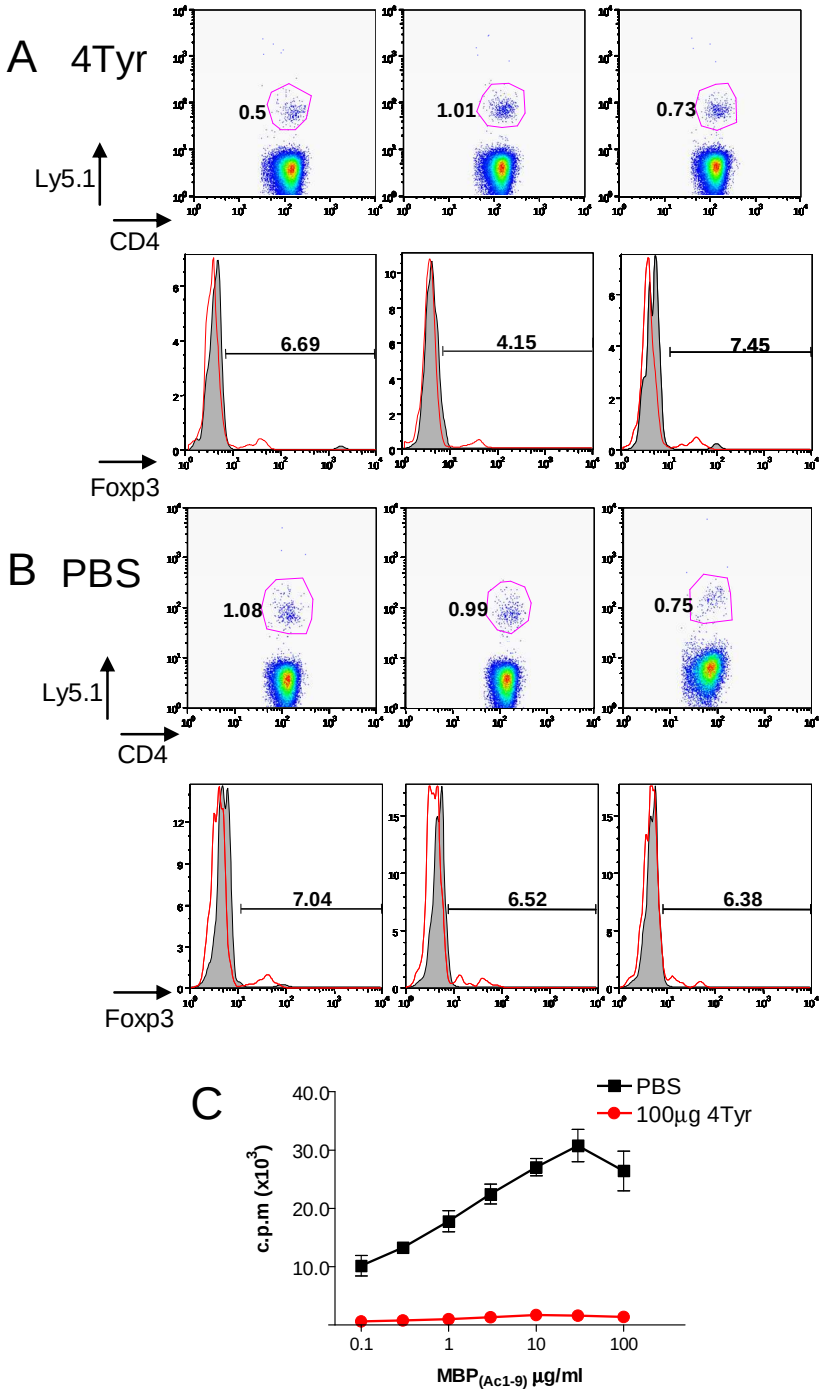
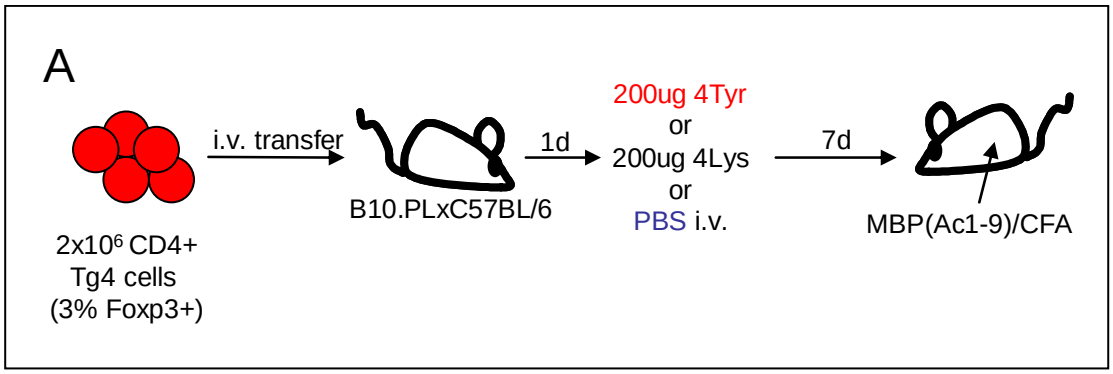


Figure 4.4: High dose Ac1-9(4Tyr) treatment results in tolerance to MBP re-stimulation after 14 days but does not increase the proportion of MBP-reactive Foxp3+ cells in vivo. B10.PL mice received 2x10^6 Tg4 CD4+ cells followed by i.v. administration of 100µg 4Tyr or PBS one day later. Spleens were harvested 14 days after peptide treatment. A: Tg4 populations (top panels) and Foxp3 expression in Tg4 cells (bottom panels) of 4Tyr treated mice. B: Tg4 populations (top panels) and Foxp3 expression in Tg4 cells (bottom panels) of PBS treated mice. Plots show three individual mice per group. C: MBP(Ac1-9)-induced proliferation of splenocytes from 4Tyr treated (○) and PBS treated mice (□). Data shown represents mean +/- S.D. of 3 mice per group, representative of two independent experiments with similar results.



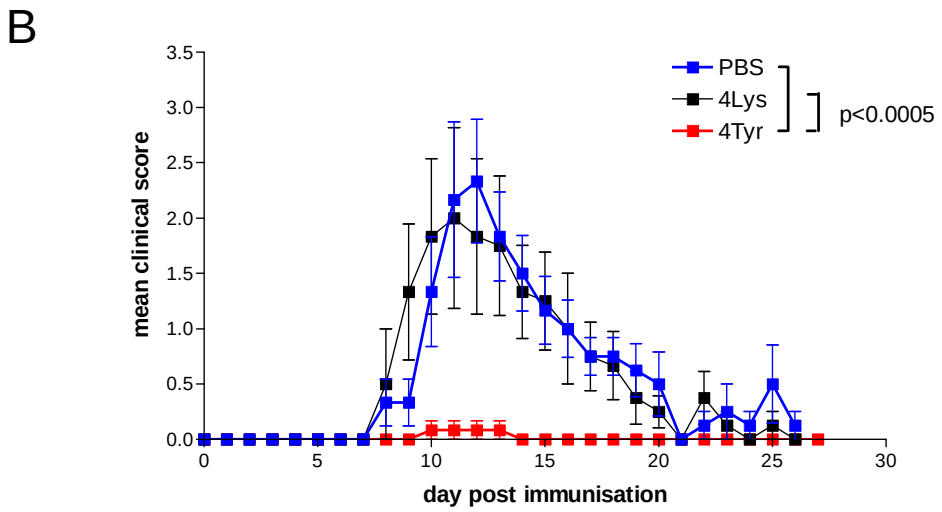


Figure 4.5: High dose pre-treatment with 4Tyr suppresses MBP-induced EAE. 2x10^6 Tg4 CD4+ cells or PBS alone was transferred i.v. to B10.PLxSJL mice. Mice were treated the following day with 200µg 4Tyr, 200µg 4Lys (WT) or PBS i.v. 7 days later, mice were immunised with MBP(Ac1-9)/CFA, plus pertussis toxin i.p x 2. A: Experimental outline. B: EAE plots. Data shown from experiment performed by J. Konkel, showing mean +/- S.E.M. of 6 mice/group. Data representative of five experiments, some performed in collaboration with J. Konkel. Statistical analysis performed using Mann Whitney test comparing mean scores per group, per day.

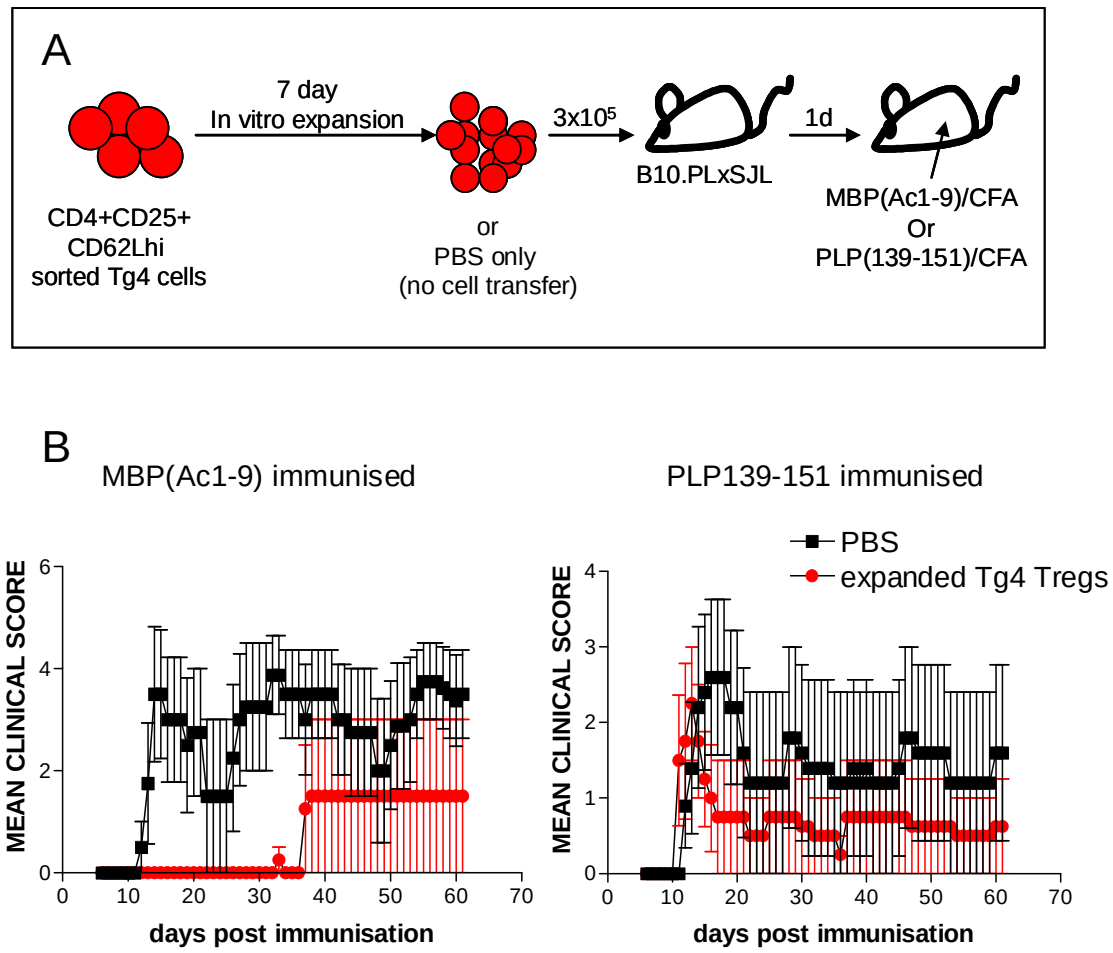


Figure 4.6: In vitro expanded Tg4 Tregs supress primary episodes of MBP-induced EAE and reduce relapses in PLP-induced EAE. A: Experimental outline – Tg4 CD4+CD25+CD62L^{hi} Tregs (>95% purity, 80% Foxp3+) were expanded in vitro for 7 days using anti-CD3/anti-CD28 coated beads + 1000U/ml IL-2. 3x10^5 expanded Tregs were transferred i.v. to B10.PLxSJL mice one day prior to EAE induction using MBP(Ac1-9) (Fig. B: left hand plot) or PLP(139-151) (Fig. B: right hand plot). Control mice received PBS i.v. at the time of cell transfer. Data shows mean +/- S.D. of 4 mice per group, representative of one of three reproducible experiments.

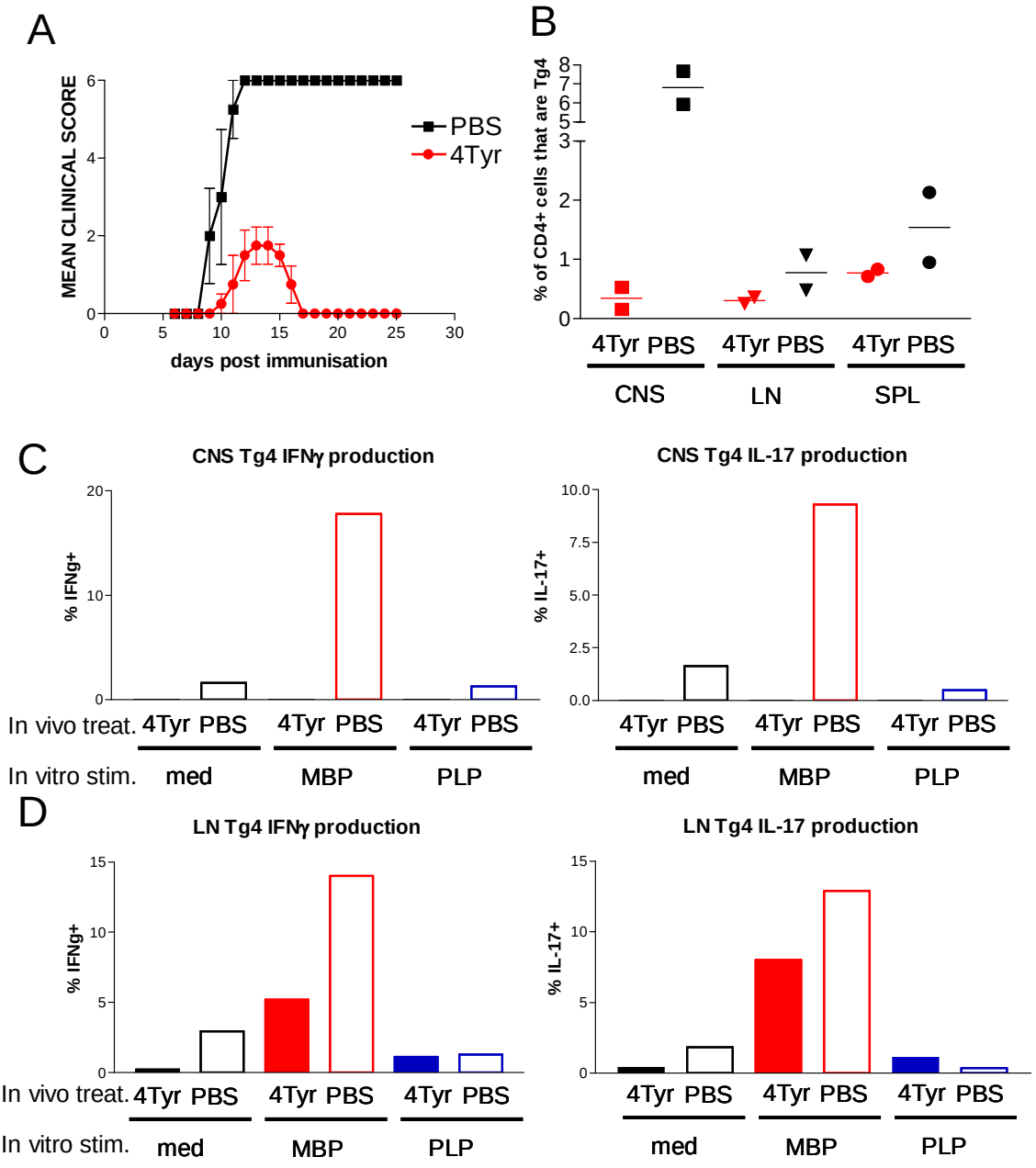


Figure 4.7: High dose 4Tyr treatment suppresses disease induced with MBP(Ac1-9) after transfer of CD4+ Tg4 cells. B10.PLxSJL mice were given 1.5x10^6 CD4+ Tg4 cells i.v. Mice were then treated with PBS or 200µg 4Tyr i.v. After 7 days mice were immunised with MBP(Ac1-9)/CFA to induce EAE. A: EAE plot of MBP-induced EAE after Tg4 transfer and treatment with 4Tyr (red line) or PBS (black line). Data shows mean +/- S.D. of 5 mice/group. B: Proportions of Tg4 cells in CNS, LN and spleen 7 days after immunisation. Data points show individual mice. C: IFNγ (left) and IL-17(right) production by Tg4 cells in CNS after in vitro stimulation with the indicated peptide. D: IFNγ (left) and IL-17(right) production in LN. Bars show pooled results of two mice per group. Data representative of two separate experiments with similar results.

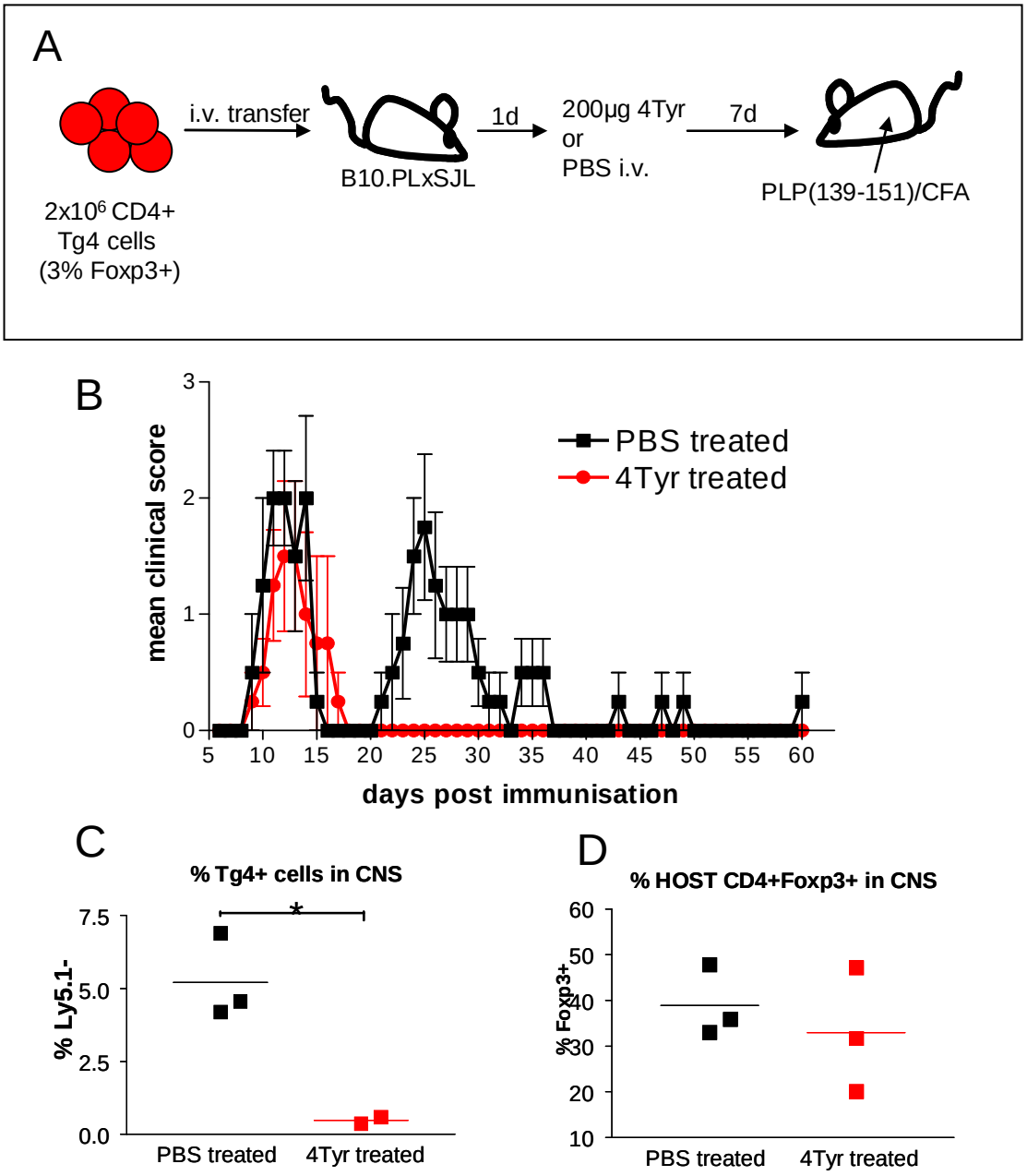


Figure 4.8: 4Tyr treatment reduces relapses in PLP-induced EAE following Tg4 cell transfer. 2x10^6 Tg4 CD4+ cells or PBS alone was transferred i.v. to B10.PLxSJL mice. Mice were treated the following day with 200µg 4Tyr i.v. 7 days later, mice were immunised with PLP/CFA, plus Ptx i.p. A: Experimental outline. B: EAE plots showing mean +/- S.D. of 6 mice per group. C and D: CNS, LN and spleen were harvested from three mice per group at day 14 of EAE. C: % transferred cells in the CNS and D: % Foxp3+ cells in total host CD4+ cells from the CNS. Data points show individual mice. * $p<0.05$. Data representative of three separate experiments with similar results.

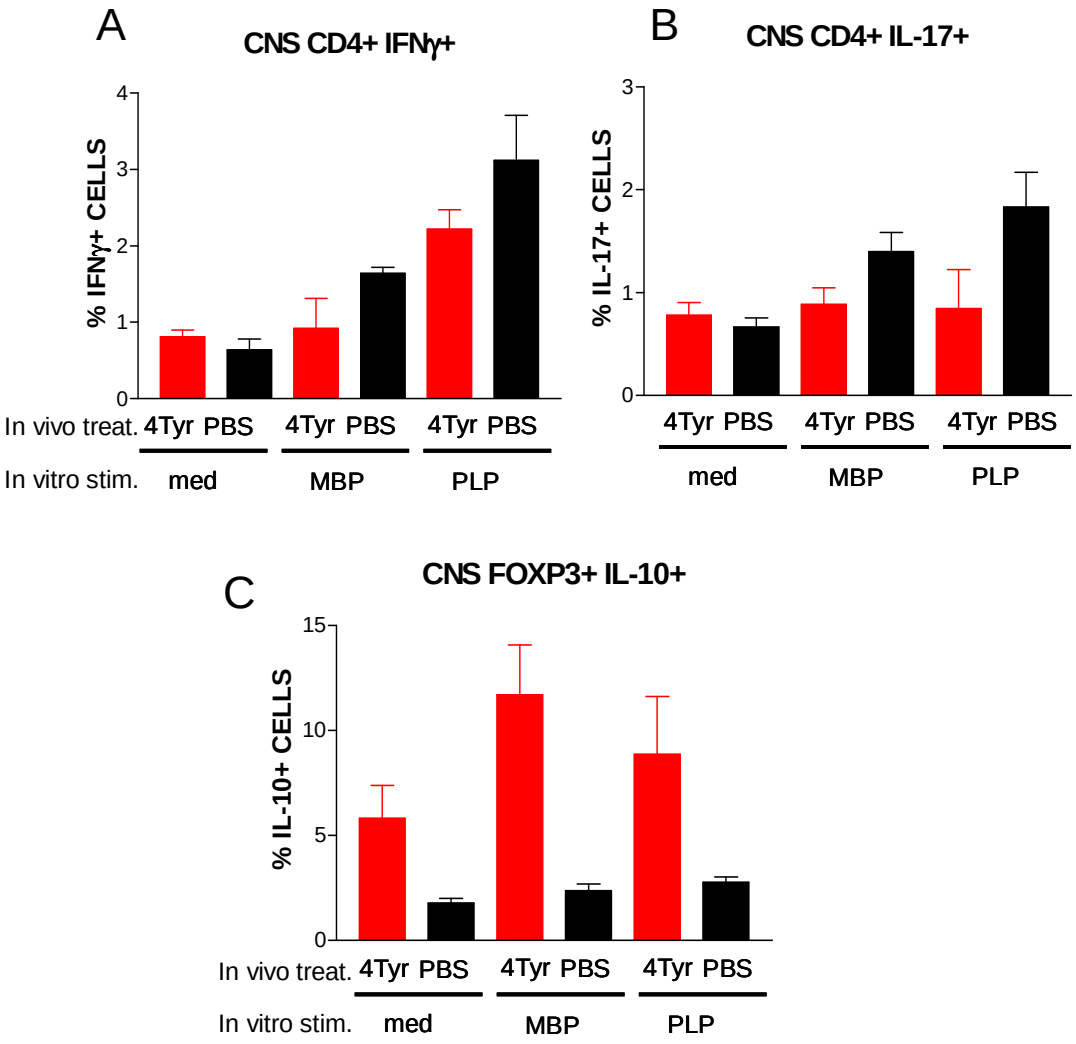


Figure 4.9: 4Tyr treatment reduced the production of effector cytokines by CD4+ cells in the CNS and increases IL-10 production. Lymphocytes from the CNS of mice from day 14 of previous figure were stimulated overnight with the indicated antigens. After 18h, BrfA was added to cultures for 4h. Total CD4+ cells were analysed for A: IFNγ, B: IL-17 and C: IL-10 intracellular cytokine production via FACS. Error bars represent 3 individual wells from pooled samples between groups. Data representative of two independent experiments.

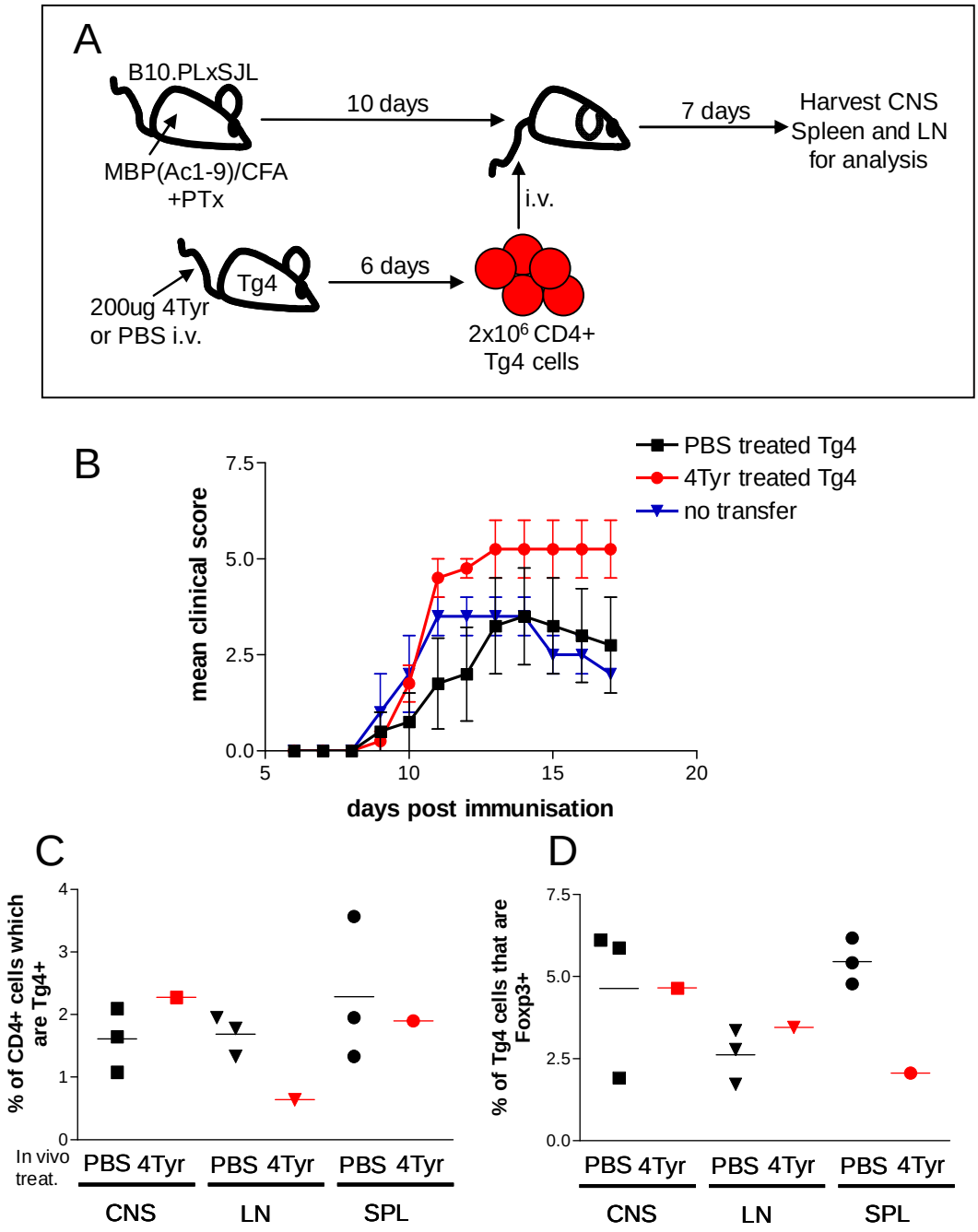


Figure 4.10: Exposure of Tg4 cells to 4Tyr in vivo prior to adoptive transfer does not suppress EAE or enhance the proportion of Tg4 Foxp3+ cells in the CNS. A: Experimental outline – Tg4 mice were given 200µg 4Tyr i.v. 6 days before CD4- purification of splenocytes. $2x10^6$ CD4+ Tg4 cells were then transferred i.v. to B10.PLxSJL mice which had been immunised with MBP/CFA 10 days earlier to induce EAE. B: EAE plots showing mean +/- S.D of three mice per group. C and D: 7 days after transfer, CNS LN and spleens were harvested to assess the proportion of Tg4 cells (C) and Foxp3+Tg4 cells (D) in each organ. Data points show individual mice and represent one of two repeat experiments with similar results.

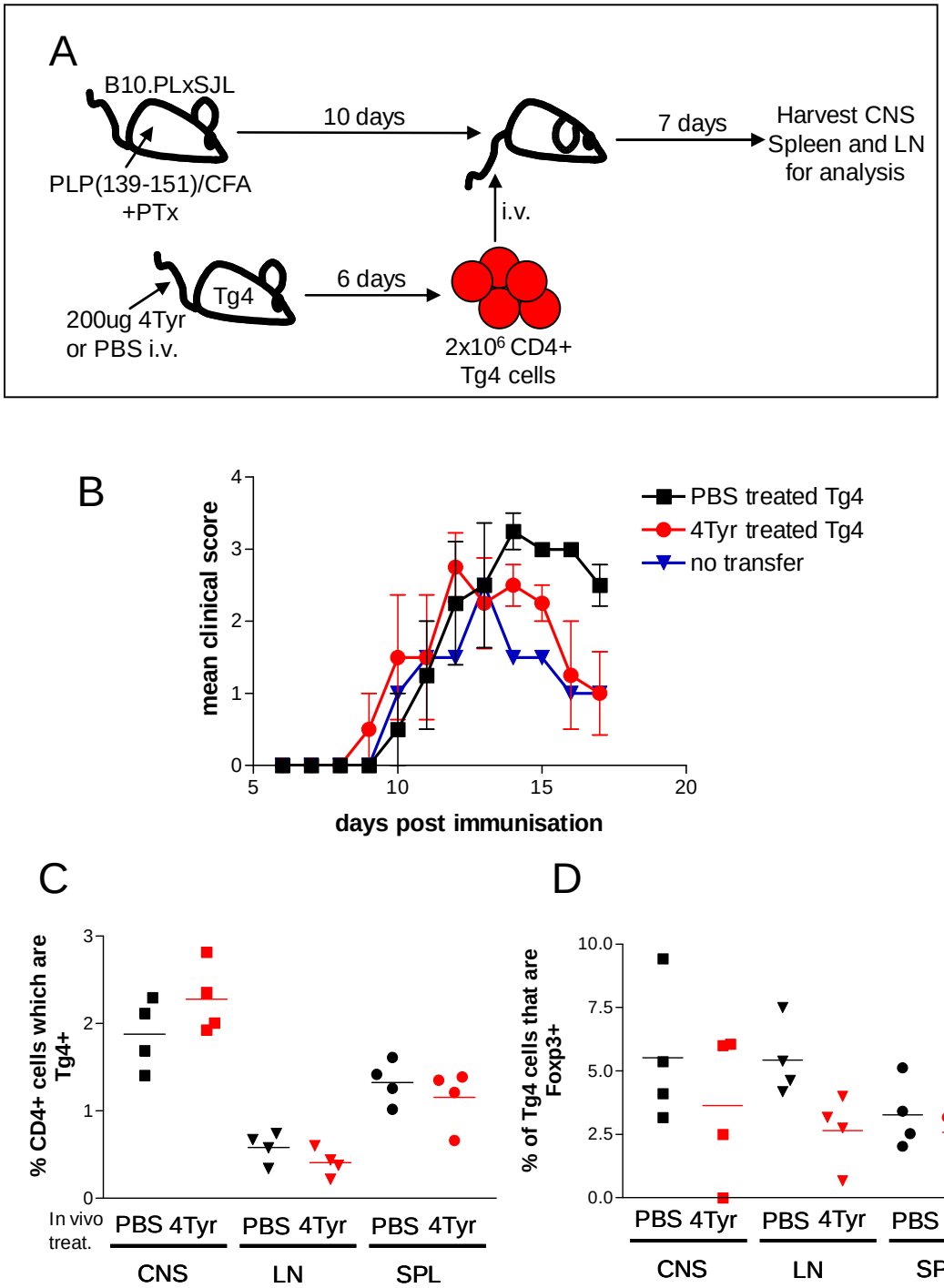


Figure 4.11: Exposure of Tg4 cells to 4Tyr in vivo prior to adoptive transfer does not suppress PLP-induced EAE or enhance the proportion of Tg4 Foxp3+ cells in the CNS. A: experimental outline. B: EAE plots showing mean +/- S.D. of four mice per group. C: Proportion of Tg4 cells in each organ. D: Proportion of Foxp3+ cells in Tg4 populations. Data points show individual mice analysed and are representative of two separate experiments with similar results.

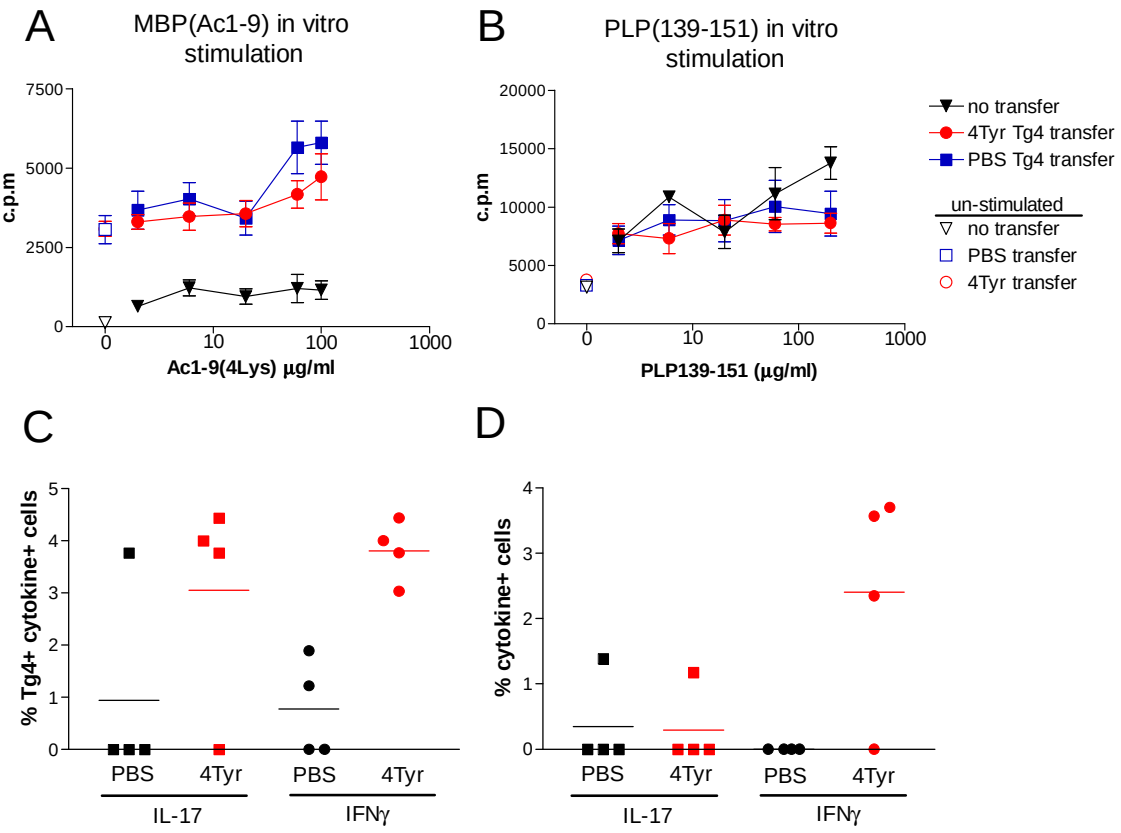


Figure 4.12: Proliferation and cytokine production of cells from PLP-immunised mice that had received Tg4 cells 10 days post EAE induction. Splenocytes from mice given 4Tyr treated Tg4 cells, PBS treated Tg4 cells or no transfer 7 days after induction of EAE with PLP(139-151) were stimulated with A: MBP(Ac1-9) and B:PLP (139-151) in vitro and proliferation assessed by ^{3}H-Thymidine incorporation for the last 18h of a 72h culture. Data shows mean proliferation +/- S.D. of 4 mice/group. C and D: IL-17 and IFNγ production by CNS derived Tg4 cells in response to MBP(Ac1-9) (C) and PLP(139-151) (D) in vitro as determined by ICCS. Data points show cytokine production by individual CNS samples, 4 mice per group. Data shows one of two repeat experiments with similar results.

5 Investigating the effect of superantigens on the peripheral Foxp3+ T cell repertoire

5.1 Introduction

There is an extensive literature on the impact of superantigens (SAg) on immunological tolerance; either as endogenous superantigens, derived from mouse mammary tumour viruses (MMTV), or as exogenous superantigens (usually staphylococcal enterotoxins (SE)) administered experimentally. For instance, the expression of particular Mtv-encoded SAg by particular mouse strains leads to the absence of T cells bearing certain TCR Vβ chains, due to clonal deletion in the thymus (Rammensee et al., 1989). Similarly, injection of SEA or SEB can be used to activate T cells bearing particular TCR Vβs, but repeated application results in their deletion via extensive activation-induced cell death (AICD), whilst those that remain are rendered unresponsive (Herman et al., 1991; O'Hehir and Lamb, 1990; Rellahan et al., 1990; White et al., 1989). Moreover there is evidence that those T cells that persist can have a regulatory function (Noel et al., 2001; Papiernik et al., 1997; Sundstedt et al., 1997). During our investigation into the TCR Vβ-usage of Tregs in naïve C57BL/6 mice we discovered that mice expressing Vβ5+ TCRs had an over-representation of Foxp3+ cells (Figure 3.17). We therefore began to investigate the mechanism behind this finding.

Towards the end of the 1980's, it was found that certain self antigens formed ligands in combination with particular products of the murine MHC, H-2, that deleted almost all T cells bearing particular TCR Vβ chains (Kappler et al., 1987; Kappler et al., 1988; MacDonald et al., 1988; Marrack and Kappler, 1988; Pullen et al., 1988). Vβ5+ T cells are deleted in mice that express mtv-3, -8, -9, -11, -17 and -30 and also express I-E, although a hierarchy exists in the capacity of each superantigen to delete Vβ+ T cells (Scherer et al., 1995). The deletion of Vβ5+ T cells has been shown to occur in mtv-6+

and -9+ mouse strains that also express I-E (Bill et al., 1990). C57BL/6 mice lack I-E and in this strain Vβ5+ cells are not deleted in the thymus, but are deleted over time in the periphery (Fink et al., 1994; Fink et al., 1992). Although I-E+ mouse strains have been reported to present SAgs more efficiently, some studies have shown that in I-E- mice (such as C57BL/6 and C57B.10 strains), I-A molecules can also present SAgs to a level sufficient to induce the chronic deletion of Vβ5+ T cells (Dyson and Elliott, 1999) and explain the findings of Fink et al. (Fink et al., 1994).

Although the stimulation of T cells by I-A/SAg occurs in I-E$^-$I-A$^+$ mice, the strength of signal must be reduced as the cells are not deleted intrathymically. Peripheral induction of regulatory T cells has been documented by groups using persistent antigen stimulation, for example using osmotic minipumps (Apostolou and von Boehmer, 2004). SAg stimulation of TCRs by mtv-derived or exogenous SAg in I-E$^-$I-A$^+$ strains may also chronically stimulate SAg-reactive T cells in the periphery. Indeed, the induction of anergic and 'regulatory' cells after superantigen stimulation has been documented by a number of groups, including the generation of CD4+CD25+ Tregs by some (Feunou et al., 2003; Grundstrom et al., 2003; O'Hehir and Lamb, 1990; Papiernik et al., 1997; Sundstedt et al., 1997); however, enhanced proportions of SAg-reactive Foxp3+ cells in the periphery had not been documented. In fact some studies suggested that Foxp3- regulatory T cells were responsible for superantigen-induced tolerance (Feunou et al., 2003).

We proposed that SAg stimulation may allow continual 'tickling' of specific Vβ+ TCRs and subsequently drive their commitment to a Foxp3+ lineage in the periphery. Alternatively, Foxp3+ T cells may have an enhanced resistance to SAg-mediated deletion in the periphery and therefore survive peripheral activation and deletion induced by chronic superantigen stimulation. We addressed these questions using un-manipulated C57BL/6 mice to assess the effect of endogenous SAg (mtv-8) on the Vβ5+ repertoire by testing the effects of low dose i.v. administration of SEB as a model for chronic SAg stimulation. The data show that SAg stimulation enhances the proportions

of Foxp3+ T cells in the periphery. This was not due to the proliferation of Foxp3+ T cells, but rather the preferential activation and deletion of Foxp3- cells, suggesting that Foxp3+ Tregs show an intrinsic resistance to SAg mediated activation and deletion. Furthermore, SAg-induced/survived Tregs suppressed the primary response of transgenic T cells to both cognate antigen and SAg stimulation in vitro, suggesting that chronic exposure to SAg will favour the balance of the immune system towards regulation. This will have important consequences on the ability to mount protective immune responses and has implications in the susceptibility to autoimmunity.

5.2 Results

5.2.1 Naïve C57BL/6 mice show elevated levels of Vβ5+Foxp3+ T cells

We have recently described a critical role for Foxp3+ Tregs in recovery from experimental autoimmune encephalomyelitis (EAE) (McGeachy et al., 2005). Several models of autoimmune disease show an oligoclonal expansion of T cells expressing TCR Vβ8 genes (Acha-Orbea, 1991) (Steinman, 2001). This is particularly notable in EAE, suggesting that the Vβ8+ T cell repertoire might have a predilection for myelin-derived self antigens. We therefore reasoned that the Vβ8+ repertoire might have a selectively higher frequency of Foxp3-expressing cells during EAE. To test this we made use of a panel of anti-Vβ antibodies to analyse Foxp3-expression in the peripheral immune repertoire. In naïve C57BL/6 mice maintained in specific-pathogen free conditions, the frequency of CD4+ cells derived from spleen or lymph nodes that expressed Foxp3 was consistently around 10%. This did not increase when we looked specifically at those cells that stained for Vβ8 (Fig. 5.1A). There was, however, a striking increase in Foxp3-expression in CD4+Vβ5+ cells, reaching a frequency of up to 1 in 3 (Fig. 5.1A and B). To determine if the over-representation of Foxp3+ cells in the Vβ5+ population was evident from the generation of Vβ5+ cell in the thymus, or if this effect was mediated by a peripheral event, we compared the Foxp3 expression of thymic Vβ5+ and peripheral (LN) Vβ5+ cells. The over-representation of Foxp3 was not evident in Vβ5+CD4+CD8- thymocytes (Fig. 5.1C). Thus, although the possibility that the selective accumulation of Foxp3+ cells in the Vβ5+ population begins in the thymus cannot be ruled out, these data suggest that it is driven chiefly in the periphery.

5.2.2 B cells play a partial role in SAg presentation to T cells in vivo

One of the initial findings that Vβ-specific deletion occurred in mice demonstrated that the antigen was B cell derived (Kappler et al., 1987). The preferential infection of B cells by MMTVs and the presentation of MMTV-derived superantigens on the surface after infection was later described (Beutner et al., 1994; Held et al., 1993).

To determine which cells may potentially be responsible for the presentation of endogenous superantigens to Vβ5+ T cells, we made use of the μMT mice (Kitamura and Rajewsky, 1992), which lack B cells, and therefore have an over-representation of T cells (Fig. 5.2A). However, the proportion of CD4+Foxp3+ cells in μMT mice was significantly reduced compared to WT mice (Fig. 5.2B). Analysis of the CD4+Vβ5+ populations in μMT vs. WT C57BL/6 mice showed that μMT mice had an increased proportion of these cells (Fig. 5.2C).

The percentage of both Vβ5+ and Vβ5- Foxp3+ cells was reduced in μMT mice (Fig. 5.2D). Because the overall proportion of CD4+Foxp3+ cells was reduced in μMT mice, we determined the ratios of CD4+, Vβ5+ and Foxp3+ cells in μMT/WT mice to identify where the differences between the strains could be found (Fig. 5.2E). These ratios confirmed that the proportion of CD4+Vβ5+ cells was indeed higher in μMT mice. This was shown to be due to the enhanced proportions of Vβ5+Foxp3- cells in μMT mice compared to WT mice, as the ratio of Vβ5+Foxp3+ cells was not enhanced (Fig. 5.2E).

The increase in Vβ5+Foxp3+ cells compared to Vβ5-Foxp3+ cells appeared less in μMT mice compared to WT (Fig. 5.2D). To assess this, the ratio of the difference from Vβ5+Foxp3+ to Vβ5-Foxp3+ between the two strains was calculated using the equation shown in Fig. 5.2F. If the absence of B cells was not having an effect on Vβ5+ T cells then the result of this equation (ratio) would be 1. However, the ratio was found to be

0.82, confirming that the fold increase from Vβ5-Foxp3+ to Vβ5+Foxp3+ cells was reduced in μMT mice compared to WT mice.

These data show that while μMT mice have a decreased frequency of total CD4+Foxp3+ cells, this is due to the enhanced proportions of Foxp3- cells in these mice and may suggest Vβ5+Foxp3- cells are not deleted as efficiently in mice which lack B cells, possibly representing a role for B cells in the presentation of endogenous superantigens in this system

5.2.3 Vβ5+foxp3- T cells show higher turnover rates in vivo

As the proportion of Vβ5+Foxp3+ cells was increased in the periphery we sought to determine if these cells were proliferating at a higher rate than other CD4+ T cells. BrdU was administered i.p. to naïve C57BL/6 mice on three consecutive days. 18 hours after the final administration spleens were harvested and the rate of turnover of the Foxp3-enriched Vβ5+ population was measured by FACS (Fig. 5.3A). The “background” level of in vivo proliferation of total CD4+Foxp3+ cells was ~4-fold higher than that of CD4+Foxp3- cells. This is consistent with similar data that used CD25 expression as a marker for Tregs to assess their turnover in vivo (Fisson et al., 2006). However, the rate of turnover in the Vβ5+Foxp3+ population was not significantly greater than that of the total CD4+Foxp3+ population as a whole, or than that of the Vβ5-Foxp3+ population. In marked contrast, the turnover rate of the CD4+Vβ5+Foxp3- population was clearly higher than that of the total CD4+Foxp3- population, and was raised to a level equivalent to that of Foxp3+ cells. These results would suggest that the Foxp3+ cells do not have an increased rate of mitosis in response to SAg stimulation and that, potentially, the increase in Treg frequency arises from AICD of the foxp3- population, although further investigation is required to confirm this theory.

5.2.4 Vβ5+ cells display enhanced levels of regulatory cell markers due to their expression by Vβ5+Foxp3+ cells

To further characterise the phenotype of Vβ5+ cells we analysed surface marker expression associated with Tregs and markers of T cell activation (Fig. 5.3B and C). Firstly, the cell surface markers on CD4+Vβ5+ compared to CD4+Vβ5+ cells were analysed (Fig. 5.3B). Vβ5+ T cells displayed a slight reduction in the levels of CD62L, while the levels of CD44 appeared similar to Vβ5- cells. The levels of 'regulatory' cell markers CD25, CTLA-4, GITR, OX-40 and CD103 appeared enhanced in Vβ5+ cells, with some markers showing distinct populations showing highest expression (CD25 and GITR) and others displaying a general shift in marker expression (CTLA-4, OX-40 and CD103). No differences were observed in the levels of CD5 or Fas (data not shown), arguing against the higher expression levels of all surface markers in Vβ5+ T cells. Next, the surface marker expression by Foxp3+ and Foxp3- populations within the Vβ5+ compartment was assessed (Fig. 5.3C). Vβ5+Foxp3+ cells displayed lower levels of CD62L but higher levels of CD44, CD25 and CD103. However, CTLA-4 expression was equal in both the Vβ5+Foxp3+ and foxp3- populations. Thus, the Vβ5+Foxp3- cells expressed marker levels associated with activation ($CD62L^{lo}CD44^{hi}$) consistent with the theory that these cells undergo activation by SAg, possibly followed by their death. The population shifts in expression of CD25 and CD103 in Vβ5+ vs. Vβ5- cells must be due to the up-regulation of these markers by both Foxp3+ and Foxp3- Vβ5+ cells, suggesting that SAg encounter is affecting the surface expression of these markers in both populations. It would be of interest to determine of SAg stimulation could also induce the conversion of Foxp3- cells towards a regulatory phenotype.

5.2.5 Vβ5+ cells have an enhanced regulatory capacity compared to Vβ5- cells in vitro

Although a higher proportion of Vβ5+ T cells expressed Foxp3, it was unclear if these cells could exert regulatory function. To determine the suppressive activity of Vβ5+ T cells, we set up an assay to test their ability to suppress the response of T effector cells in vitro (Fig. 5.4). CD4+ cells were sorted from naïve C57BL/6 mice by MACS-purification, followed by Vβ5+, Vβ5- and CD25+ sorting to generate 'suppressor' cell populations. CD25- cells were sorted as 'effector' cells and the foxp3-expression of each population assessed prior to culture (Fig. 5.4A). CD4+CD25+ populations were ~90% Foxp3+, while CD25- effector cells were ~4% Foxp3+. Consistent with previous data, Vβ5+ populations had an approximate 4-fold increase in the proportion of Foxp3+ cells compared to Vβ5- populations (~30% vs. ~8% respectively, Fig. 5.4A). Effector cells were cultured with various ratios of suppressor cells and stimulated with anti-CD3 in vitro for 48 before analysis of proliferation by addition of 3H-Thymidine for the final 18 hours of culture. The suppression of responder cells was compared to their culture with anti-CD3 in the absence of suppressor cells. Vβ5+ cells were as suppressive as sorted whole CD25+ cells when cultured with CD25- cells at high ratios (Fig. 5.4B). At lower ratios of Treg:Teff, Vβ5+ cells lost the capacity to suppress, probably due to the dilution of Tregs in culture (Fig. 5.4B). Vβ5- cells used as a control did not show suppression at any ratio (Fig. 5.4B). This showed that encounter with endogenous SAg skewed SAg-reactive populations towards a regulatory phenotype that was capable of suppressing the proliferation of polyclonal effector T cells in vitro.

5.2.6 Exposure to low dose SAg activates CD4+Vβ3+ and CD4+Vβ8+ T cells in vitro

The data described above would imply that encounter with SAg skews the T cell population towards the regulatory, Foxp3-expressing component. We reasoned that if

this was the case, we should be able to reproduce this effect experimentally by giving exogenous superantigens. Exposure to the staphylococcal enterotoxins SEA and SEB is a well established method inducing immune tolerance in T cells expressing the appropriate Vβ genes (Fink et al., 1994; Lobo-Yeo and Lamb, 1993). This tolerance results from a high level of AICD, coupled with anergy in those T cells that survive (Lobo-Yeo and Lamb, 1993; Rellahan et al., 1990; White et al., 1989) . Although regulatory/suppressive functions have been described in these remaining cells, such as a dominant production of IL-10 (Noel et al., 2001; Sundstedt et al., 1997), the over-representation of foxp3 has not been documented. In fact, there is evidence to the contrary (Feunou et al., 2003). SEB has been reported to activate T cells expressing either Vβ3 or Vβ8 (Rellahan et al., 1990; White et al., 1989). Similar to endogenous superantigens, SEB was shown to have differential stimulatory effects in mouse strains with different MHC backgrounds and SEB presentation was shown to be more effective by I-E than I-A molecules (White et al., 1989).

To confirm that SEB would activate T cells bearing specific TCR Vβ chains, we cultured naïve splenocytes from C57BL/6 mice with various doses of SEB or with con A, as a control for T cell activation irrespective of Vβ-usage (Fig. 5.5A). After stimulation with con A, the frequencies of T cells expressing either Vβ3, Vβ8, or Vβ6 (used as a control) remained equivalent to those found directly ex vivo (Fig. 5.5A, compared with Fig. 5.1A). In contrast, culture with SEB led to a two- to three-fold increase in the Vβ8 frequency and a five-fold increase in the Vβ3 frequency. As a reflection of this expansion of Vβ3 and Vβ8 T cells, the frequency of Vβ6+ cells dropped by around 90% (Fig. 5.5A).

5.2.7 SEB treatment enhances frequency of SEB-reactive Foxp3+ cells in vivo.

We next tested the effects of SEB on Vβ3 and Vβ8 expressing cells in vivo. Three or five intravenous doses of 1 µg SEB or PBS were administered over a five-day period to

naïve C57BL/6 mice (Fig. 5.6A). 24 hours after the last treatment, splenocytes were harvested from each mouse to assess the levels of Vβ3+ and Vβ8+ cells ex vivo. Vβ6 staining was used as a non SEB-reactive TCR Vβ control. Treatment with 3 or 5 doses of 1μg SEB resulted in the depletion of CD4+ cells that expressed Vβ3 or Vβ8. This was coupled with an increase in Vβ6+ T cells (Fig. 5.6B, left panels). FACS analysis of Foxp3-expression within the Vβ+ populations showed that SEB administration increased the frequency of Foxp3+ cells amongst Vβ8+ cells from 10 to 15%, in contrast to the comparatively stable expression in Vβ6+ cells (Fig. 5.6B). More marked was the increase in cells that expressed Foxp3 amongst Vβ3+ cells, increasing to levels approaching 50% (Fig. 5.6B, right panels). This increase in Foxp3 expression specifically amongst SEB-sensitive T cells could be accounted for by three processes; either specific proliferation of Foxp3-expressing cells, their resistance to AICD compared with their Foxp3- counterparts, or de novo activation of foxp3.

To test these possibilities we gave C57BL/6 mice three doses of SEB over five days and also gave BrdU every two days to provide a measure of mitosis over this period (Fig. 5.7A). In confirmation with previous data, Vβ3+ T cells were depleted in response to SEB stimulation, while Vβ6+ cells were unaffected by SEB stimulation (Fig. 5.7B). The loss of total Vβ3+ cells was associated with an increase in the proportion of Vβ3+Foxp3+ cells (Fig. 5.7C). In PBS treated control mice, the proportion of CD4+Foxp3+ cells that were cycling was approximately two-fold higher than their Foxp3- counterparts (Fig. 5.7D). In mice that had received SEB i.v., the level of proliferation in the Vβ6+ population remained higher in the Foxp3+ fraction (Fig. 5.7D). In contrast, in the SEB-sensitive Vβ3+ population, the level of BrdU incorporation was raised in the Foxp3- fraction to a level equivalent to, or higher than, the Foxp3+ fraction (Fig. 5.7D). Furthermore, the level of BrdU incorporation in the Vβ3+Foxp3+ fraction was not increased in mice that had received SEB. This lack of enhanced in vivo proliferation amongst superantigen-sensitive Foxp3+ cells is supported by BrdU data from SEB-stimulated in vitro cultures which also did not show incorporation by SEB-sensitive Foxp3+ cells (data not shown). These data suggest that the decrease in

CD4+Vβ3+ T cells in response to SEB stimulation is due to the activation, proliferation and death in Vβ3+Foxp3- cells, while the Foxp3+ fraction remains unresponsive to SEB stimulation.

5.2.8 SEB-stimulated transgenic T cells are anergic to TCR mediated stimulation in vitro

The ability of T cells which have experienced SEB to respond in vitro was tested using a TCR transgenic mouse model. All T cells in Tg4 transgenic mice are reactive to only one peptide, MBP(Ac1-9) and express only one TCR Vβ gene, encoding Vβ8. Every T cell in these mice therefore has the ability to respond to SEB. After exposure to SEB (3 doses of 1ug SEB, over three consecutive days) or PBS, splenocytes were harvested and assessed for foxp3 expression (Fig. 5.8A). As was seen in the C57BL/6 mice given SEB, Tg4 mice showed elevated frequencies (approximately 3-4 fold increase) of Foxp3+ cells (Fig. 5.8B, top panels). This was a greater increase than seen in the Vβ8+ population using SEB in C57BL/6 mice (Fig. 5.6). Interestingly, the proportion of Foxp3+ cells expressing CD25 drops dramatically when stimulated with SEB (Fig. 5.8B, bottom panels) which may explain the finding that both CD25+ and CD25- populations from SEB-treated mice are suppressive (Grundstrom et al., 2003). We next tested splenocytes from SEB or PBS treated Tg4 mice for a response to both SEB and MBP(Ac1-9) in vitro (Fig. 5.8C). Splenocytes from mice exposed to SEB in vivo showed very little or no proliferation to either SEB or MBP(Ac1-9) in vitro compared to PBS-treated mice; i.e. SEB-experienced Tg4 cells were tolerant both to SAg and to their cognate peptide antigen.

Supernatants from the stimulated cell cultures were also assessed for the levels of IL-2 and IFNγ by ELISA (Fig. 5.9A). This revealed a profound reduction in IL-2 production by SEB-treated splenocytes after re-stimulation in vitro with either SEB or MBP(Ac1-9) (Fig. 5.9B). The overall levels of IL-2 detected in SEB-stimulated control cultures were

lower than those seen in MBP(Ac1-9)-stimulated control cultures. The levels of IFNγ produced by cells from SEB-treated mice were also reduced compared to cells from PBS-treated mice (Fig. 5.9B). No IL-10 could be detected in the supernatants by ELISA (data not shown). These data were confirmed using intracellular cytokine staining for IFNγ and IL-10 after overnight stimulation with SEB or (Ac1-9) (Fig. 5.9C). IFNγ production in response to SEB stimulation was reduced in SEB-experienced splenocytes compared to splenocytes from PBS-treated mice, and also marginally reduced in MBP(Ac1-9)-stimulated cultures. Very little IL-10 could be detected in any cultures and there was no significant change in IL-10 levels in SEB- vs. PBS-treated mice (Fig. 5.9C).

5.2.9 SEB stimulated Tg4 cells suppress naïve Tg4 T cell responses to SEB and MBP(Ac1-9) in vitro

The proportion of Foxp3+ cells in SEB-treated CD4+ Tg4 cells was ~4-fold higher than in PBS-treated mice (Fig. 5.8B). We next sought to determine whether these SEB-experienced cells were also suppressive. Using a co-culture assay, suppressive capacity of naïve T cells vs. T cells from SEB-treated mice could be assessed. Sorted CD4+ cells (MACS CD4-positive selection) from SEB- or PBS-treated Tg4 Thy1.1- mice were cultured in vitro with CD4+ cells from Thy1.1+ naïve Tg4 mice in the presence of irradiated APC (B10.PL splenocytes) and SEB or MBP(Ac1-9) (Fig. 5.10A). By adding BrdU for the final 18h of culture the proliferation of cells derived from naïve and treated mice in co-culture could be distinguished by Thy1.1 FACS. Co-staining of BrdU and Foxp3 allowed us to analyse the proliferation in Foxp3- cells and Foxp3+ cells individually.

CD4+ populations from PBS-treated and naïve mice were ~97% foxp3-, regardless of the stimulus used (data not shown). When cultured without the addition of SEB-experienced cells, one third of naïve or PBS-treated Foxp3- cells incorporated BrdU in

response to MBP(Ac1-9) and almost 50% responded to SEB (Fig. 5.10B and C). Reiterating the data shown in Fig. 5.8B, CD4+ splenocytes from SEB-treated mice showed reduced BrdU incorporation by Foxp3- cells to both MBP(Ac1-9) and SEB (only 10.5% and 3.8% respectively) (Fig. 5.10B), presumably due to the increased frequency of Foxp3+ cells in these cultures. When in co-culture with cells from SEB-treated mice, the proliferation of naïve Foxp3- cells in response to SEB was greatly diminished compared to when cultured alone, or with PBS-treated Tg4 cells, from ~50% to ~10% respectively (Fig. 5.10C). The proliferation of naïve Foxp3- cells in response to MBP(Ac1-9) was also suppressed by co-culture with SEB-experienced cells, although this effect was less pronounced (Fig. 5.10C). When co-cultured with cells from PBS-treated mice, the proliferation of naïve Foxp3- cells was enhanced in response to both SEB and MBP(Ac1-9) stimulation (Fig. 5.10C).

As FACS staining allowed discrimination of Foxp3+ and Foxp3- cells we could also study the effects of in vitro stimulation on Treg proliferation. When cultured alone, cells from naïve or PBS-treated mice did not show enhanced BrdU incorporation in response to SEB or Ac1-9 (Fig. 5.10D and E). However, SEB-experienced Foxp3+ cells did increase BrdU incorporation when cultured with SEB (but not when cultured with Ac1-9) (Fig. 5.10D). Moreover, in co-cultures, SEB-experienced cells promoted a three-fold higher incorporation of BrdU in naïve Foxp3+ cells (Fig 5.10E). Note that this was also at the same time as suppressing BrdU incorporation in naïve Foxp3- cells from 32% to 13% (Fig. 5.10C).

These results suggested that T cells from SEB-treated mice TCR transgenic mice induced the suppression of naïve transgenic T cell proliferation in response to SEB and their cognate antigen, MBP(Ac1-9). Furthermore, stimulation of naïve T cells in the presence of SEB-experienced cells increased the proliferation of naïve Foxp3+ cells, although this effect was only seen upon in vitro stimulation with SEB and not when MBP(Ac1-9) was used, suggesting a unique effect of SEB re-stimulation on SEB-experienced cells. This may be via the release of IL-10 or TGFβ by SEB-experienced

cells. The levels of cytokines in the supernatants of the co-culture experiments were assessed by ELISA. Co-culture of naïve T cells with SEB-experienced T cells reduced the levels of IL-2 (Fig. 5.11A) and IFNγ (Fig. 5.11B) but enhanced the level of IL-10 (Fig. 5.11C) produced in response to SEB stimulation. These data suggest that the suppression observed in SEB stimulated co-cultures may be due to the secretion of IL-10 by the SEB-experienced cells. This would require further testing using blocking anti-IL-10 antibody.

5.2.10 The effect of prior exposure to low dose SEB on the induction EAE using Tg4 TCR transgenic mice

As Tg4 mice express a disease relevant TCR we sought to determine the effect of SEB-stimulation on EAE induced using MBP(Ac1-9) in Tg4 mice. 1μg of SEB was administered i.v. directly to Tg4 mice on three consecutive days prior to induction of EAE using MBP(Ac1-9) one day after the final treatment (Fig. 5.12A). Tg4 mice that received SEB prior to disease induction were protected from disease (Fig. 5.12A). Mice that received PBS developed clinical signs of EAE, although the incidence of disease was low in this experiment (50%). This suggested that SEB administration may have had an effect on the encephalitogenic potential of the MBP(Ac1-9) reactive cells.

It had previously been shown that B10.PLxC57BL/6 mice require the adoptive transfer of Tg4 cells in order to develop disease (McCue et al., 2004). We therefore transferred $2x10^6$ Ly5.1+ Tg4 cells into Ly5.1- B10.PLxC57BL/6 host mice to track the population of cells that were responsible for disease induction. Mice then received either 1μg SEB or PBS i.v on three consecutive days (Fig. 5.12B). As Ac1-9(4Tyr) had previously been shown to have profound tolerogenic properties in this model (Fig. 4.5), one group of mice received 200μg Ac1-9(4Tyr) one day after adoptive transfer of Tg4 cells as a control to compare the tolerogenic potential of SEB. On the fourth day after T cell transfer all mice were immunized with MBP(Ac1-9)/CFA to induce EAE (Fig. 5.12A).

Mice that had received 3 doses of SEB after adoptive transfer of CD4+ Tg4 cells showed reduced disease incidence and severity compared to mice that received PBS (Fig. 5.12B). However, the tolerance induced by SEB was not as profound as that seen with Ac1-9(4Tyr) administration. All mice were sacrificed at day 17 to assess the effect of SEB on the Tg4 population in the CNS, draining lymph nodes and spleen, however no Tg4 cells could be detected in this experiment.

5.3 Discussion

Data presented in this chapter show that exposure to superantigen has a major role in determining the frequency of Foxp3+ T cells in the peripheral immune repertoire. Superantigens have been known for some time to have profound immunomodulatory properties (Herman et al., 1991; Lobo-Yeo and Lamb, 1993). However, this study shows a positive association with Foxp3, a key transcription factor associated with immune tolerance, such that the frequency of superantigen-reactive Foxp3+ cells is enhanced upon superantigen exposure. This seems to be through loss of foxp3- cells rather than a positive expansion of foxp3+ cell numbers. Furthermore, supernatigen-induced or 'spared' T cells were capable of suppressing the primary response of naïve T cells in vitro. This was the case in both mmtv-induced Vβ5+ T cells suppressing anti-CD3-driven proliferation of CD25- cells (Fig. 5.4) and of SEB-induced Tg4 T cells suppressing naïve Tg4 cell responses to both MBP(Ac1-9) and SEB (Fig. 5.9). Although encounter with superantigen skewed the SAg-reactive population towards Foxp3+ cells, it cannot be ruled out from these experiments that the Foxp3- cells remaining after SAg-encounter contribute to the suppression observed. This would require sorting of SEB-experienced Foxp3+ and Foxp3- cells as can now be done using Foxp3-GFP+ mice (Fontenot et al., 2005b).

The data showing the effects of both endogenous and exogenous superantigen indicate that superantigen-reactive T cells are activated in vivo, with Foxp3- cells turning over at a faster rate then Foxp3+ cells. In the naïve setting, Vβ5+ cells are relatively rare in the total CD4+ population (3-5%, Fig. 5.1B and 5.3B) and after administration of SEB, the total CD4+Vβ3+ and CD4+Vβ8+ populations are reduced. These results would suggest that Foxp3- T cells bearing the appropriate TCR Vβ are activated and eventually undergo AICD when they encounter SAg in vivo. In contrast, those Vβ-expressing T cells that are Foxp3+ are spared this fate and as a result reach significant frequencies in

the overall Vβ+ population. Indeed the avoidance of AICD by T cells expressing Foxp3+ had previously been suggested (Papiernik et al., 1998).

A recent study has used the thymic selection and chronic deletion of Vβ5+ T cells in C57BL/6 mice as a model to assess the capacity of Tregs to be deleted by TCR-mediated apoptosis (Taylor et al., 2007). This group proposed that as SAg-reactive T cells are not deleted in the thymus of these mice due to the lack of I-E molecules, then these cells should have a high avidity towards self and be selected close to the level of stimulation that would normally induce negative selection. As Vβ5+ cells show a skewing toward Foxp3-expression, this may be explained by the preferential selection of high avidity self-reactive T cells to become Foxp3+ cells in the thymus. However, similar to our data, it was shown that positive selection of Vβ5+Foxp3+ and Foxp3- cells occurred with a similar efficiency, regardless of the avidity for self antigen. The skewed frequency of Vβ5+Foxp3+ cells was shown to be due to the selective resistance of Foxp3+ cells in response to both thymic and peripheral SAg stimulation (Taylor et al., 2007), in agreement with the data presented in this chapter.

Of note, regarding superantigen-mediated deletion of transgenic T cells, OT-II mice that express a Vα2+Vβ5+ TCR and are on the C57BL/6 background (mtv-8+) do not undergo TCR revision or show deletion of CD4+ T cells (Zehn et al., 2007). This suggests that an aspect of this transgenic mouse strain prevents the stimulation of the transgenic T cells by endogenous supreantigens, although the mechanism behind this is unknown

The ability of bacterial superantigens to induce the activation of Vβ-specific T cells, followed by the deletion of the majority of these cells (and those remaining being anergic), has long been known (Kawabe and Ochi, 1990; Kawabe and Ochi, 1991; MacDonald et al., 1991). Furthermore, data from numerous groups have shown that repeated exposure to low dose superantigen can prevent superantigen-induced shock (Feunou et al., 2003; Florquin and Goldman, 1996; Grundstrom et al., 2003; Miller et

al., 1999; Noel et al., 2001; Sundstedt et al., 1997). Similar data had also been shown for the T cells that remain after MMTV-mediated deletion in vivo (Papiernik et al., 1997) and suggest that active regulation may be responsible for SAg-induced tolerance. Other studies reported that superantigen challenge was associated with a decrease in IL-2 and IFNγ production and unchanged, or enhanced production of IL-4, IL-5 and IL-10 (Florquin and Goldman, 1996; Noel et al., 2001), suggesting that Th2 deviation was responsible for SAg-induced tolerance (Kuschnaroff et al., 1999).

Using i.p. administration of SEB, Fenou et al. (Feunou et al., 2003) found that while natural Tregs were responsible for the prevention of excessive cytokine release in response to superantigen challenge, the tolerance observed post SEB-treatment was due to a CD4+CD25-Foxp3- population via the secretion of IL-10. A similar study by Braun and colleagues (Noel et al., 2001) found a role for CD4+IL-10+ cells in the suppression of naïve T cell responses to superantigen. Importantly, these studies suggest that the mechanisms responsible for anergy in response to SAg stimulation is distinct from the suppression of SAg induced stimulation. The former may involve nTregs, as shown in RAG-/- mice where SAg-induced tolerance is reduced compared to RAG-sufficient hosts (Grundstrom et al., 2003), while the latter scenario seems to depend on IL-10 and perhaps TGFβ (Feunou et al., 2003; Noel et al., 2001). Sundstedt et al. also reported a role for CD4+ IL-10 producing cells in SAg-induced tolerance (Sundstedt et al., 1997), however this study was performed prior to the identification of Foxp3 as a nTreg marker.

A study in the late 90's by Papiernik et al. (Papiernik et al., 1997) suggested that after infection with viral mmtv, the chronic stimulation of Vβ6+ T cells by virally-encoded superantigen spared a population of CD4+CD25+ cells that produced IL-10. Similar to our data, it was shown that while the total SAg-reactive Vβ6+ T cells were reduced, this was due to the loss of Vβ6+CD25- cells as the levels of Vβ6+CD25+ cells remained constant and these cells did not proliferate. This is somewhat strange since, although Tregs show profound anergy in vitro, there is now considerable evidence that they are

capable of clonal expansion in vivo in response to a range of antigenic stimuli (Klein et al., 2003). Thus it seems that Tregs can expand to antigen, but not to superantigen. This does, however, make some evolutionary sense. If the response to superantigen was for Foxp3- cells to die and Foxp3+ cells to selectively expand, then, over time and repeated superantigen exposure, the T cell repertoire would become skewed heavily towards the Treg phenotype. This could have profound consequences for the ability to fight infection effectively. Our data show that while primary exposure to superantigen does not induce the proliferation of SAg-reactive Tregs, re-stimulation in vitro could drive the proliferation of some of these cells (Fig. 5.9B). However, re-stimulation did not induce the proliferation of the Foxp3- cells in culture (Fig. 5.9D). This suggests that while one mechanism to induce tolerance to superantigen re-exposure is the selective survival of Foxp3+ cells, the superantigen-reactive Foxp3- cells that remain are also refractory to further stimulation.

The co-culture of naïve Tg4 cells with SEB-experienced Tg4 cells demonstrated the profound suppression of naïve Foxp3- cell proliferation in response to SEB or MBP(Ac1-9) stimulation (Fig. 5.9B). When re-stimulated with SEB, Foxp3+ cells from SEB-treated mice showed enhanced proliferation compared to cells from PBS-treated mice and naïve mice. Moreover, Foxp3+ cells from naïve mice were induced to proliferate in response to SEB when in the presence of SEB-experienced cells (Fig. 5.9E). This suggests that prior exposure to SEB induces a proportion of cells that, when re-stimulated with SEB, can be induced to expand and induce expansion of naïve Foxp3+ T cells, perhaps reminiscent of infectious tolerance (Andersson et al., 2008; Cobbold and Waldmann, 1998). The enhancement of Foxp3+ cell proliferation will likely contribute to the suppression of Foxp3- cells. It is notable that the proliferation of SEB-experienced and naïve Foxp3+ cells does not occur in MBP(Ac1-9) stimulated cultures, however, the Foxp3- cells in these cultures were still suppressed, suggesting that proliferation of the Foxp3+ compartment is not necessary for their suppressive function in this assay.

One explanation for the reduced proliferation of SEB-experienced Foxp3- cells may be that SEB had induced TCR rearrangements and skewed the TCR away from the transgenic Vα4.2+ (as these mice are not RAG-/-) making these cells un-responsive to MBP. TCR revision has been described as a mechanism whereby T cells that are chronically stimulated with antigen can escape AICD. Indeed this was shown to be the case in Vβ5-expressing transgenic mice upon their encounter with mmtv in the periphery (Blish et al., 1999; McMahan and Fink, 2000). A recent study by Zehn et al. (Zehn et al., 2007) has investigated TCR revision in Vβ5+ T cells in response to mmtv-derived superantigen stimulation. This study found that Vβ5+Foxp3- cells routinely revised their TCR to express another Vβ-chain, while Vβ5+Foxp3+ cells did not undergo TCR revision and were not deleted to the same extent as their Foxp3- counterparts. This could explain the reduced response of Foxp3- (but not the Foxp3+) SEB-experienced Tg4 cell to SEB re-stimulation in vitro (Fig. 5.9B vs. D). To test if TCR revision is responsible for the reduced responsiveness of SEB-treated CD4+Foxp3- cells the levels of Vβ8 on the Tg4 cells could be assessed after SEB administration.

It would seem unlikely that all cells stimulated with SEB would lose the transgenic Vβ chain, therefore this cannot completely explain the observed unresponsiveness to MBP(Ac1-9) and SEB by SEB-experienced Foxp3- cells in vitro. Furthermore, this would not explain the reduced response of naïve Foxp3- cells in response to MBP(Ac1-9) or SEB (Fig. 5.9C) and argues for the active regulation by SEB-experienced T cells in culture. To confirm this it would be possible sort Foxp3- cells away from Foxp3+ cells and assess if they remain refractory to superantigen stimulation. This would determine if their un-responsiveness is a cell intrinsic mechanism or due to the suppression exerted by the SAg-induced Tregs.

It is possible that re-stimulation of SEB-experienced cells in vitro causes release of suppressive and Foxp3-inducing cytokines which would affect the naïve T cell response. Indeed, in these co-culture experiments IL-10 could be detected in SEB-experienced/naïve cell co-cultures by ELISA (Fig. 5.11), but not in any of the single

cultures (Data not shown). TGFβ is another potential regulatory cytokine known to induce Tregs. A recent study has suggested that Treg -derived TGFβ is responsible for Treg-mediated infectious tolerance as this cytokine can induce the conversion of naïve cells towards a Foxp3+ cell with regulatory capacity (Andersson et al., 2008). The role of this cytokine in the experiments described here is yet to be identified with the use of blocking TGFβ-antibody.

The deletion of all TCRs bearing particular TCRβ chains by superantigens will have profound consequences on the peripheral T cell repertoire. These may include the deletion of potentially protective pathogen-responsive T cells, to the beneficial removal of self-reactive T lymphocytes. The preferential use of the TCR Vβ gene segment encoding Vβ8 has been documented in EAE (Urban et al., 1988) and because SEB has been shown to stimulate T cells bearing this Vβ chain, it is unsurprising that the effect of SEB on EAE has been well studied. Our studies have shown that SEB administration can reduce disease induced with MBP(Ac1-9) in Tg4 transgenic and B10.PLxC57BL/6 mice. Other studies have shown that the administration of one high dose of SEB (100μg i.p.) prior to disease induction with Ac1-9 was shown to reduce clinical signs of EAE (Soos et al., 1993). However, SEB was also demonstrated to induce relapses in MBP-induced EAE when given after the primary episode of disease (Brocke et al., 1993). Das et al., also reported that disease was exacerbated when SEB was given prior to EAE induction by immunization with PLP (Das et al., 1996), suggesting the effect of SEB depends on the diversity of the T cell repertoire and likely the frequency of SAg-reactive cells in the population.

The important question that arises from these studies is how superantigens impact on humans. The expression of endogenous superantigens in humans has been identified, derived from human endogenous retroviruses (HERVs) (Nelson et al., 2004). It was identified that infection with EBV could transactivate HERV-K18 that encodes a superantigen able to stimulate Vβ7+ and Vβ13+ T cells (Sutkowski et al., 2001). One study, using HLA class II transgenic mouse strains has demonstrated that exposure to

endogenous human superantigens can induce thymic deletion of T cells bearing SAg-reactive TCRs and reduce the capacity of these mice to respond to SAg in the periphery (Rajagopalan et al., 2005). It would be interesting to determine if these mice also have reduced capacity to respond to other (non-super) antigens also. These studies suggest that, similar to mice, endogenous superantigens may have the capacity to profoundly influence the T cell repertoire in humans.

Interestingly, two studies have suggested that exposure to bacterial superantigen is detrimental to Treg function. Cardona et al. (Cardona et al., 2006) reported the upregulation of GITRL on monocytes when human PBMCs were stimulated with SEB in vitro. As ligation of GITR has been shown to block the function of Tregs (McHugh et al., 2002; Shimizu et al., 2002) the capacity of Tregs to suppress after SEB stimulation was shown to be reduced (Cardona et al., 2006). The chronic inflammatory skin disease atopic dermatitis (AD) is associated with colonization of the skin by S. aureus (Hauser et al., 1985; Leyden et al., 1974). A study by Ou et al. has assessed the number and function of Tregs in AD patients and showed that AD patients have enhanced levels of CD4+CD25+ T cells. However, while the Tregs from these patients could suppress anti-CD3 driven T cell response, the AD Tregs did not suppress SEB driven proliferation. The clarity of these studies is clouded by the fact that suppression of the CD25- cells in culture with CD25+ cells from AD patients was measured by 3H-Thymidine incorporation. As the data in our studies show, and by the admission of the investigators in the paper, SEB re-stimulation induces the proliferation of SEB-experienced Tregs, suggesting that the suppressive capacity of Tregs in AD may not be reduced and requires further investigation.

The effect of superantigens on autoimmunity was identified almost twenty years ago, while investigating the observation that mice expressing I-E were protected from diabetes (Nishimoto et al., 1987). Reich et al. reported that diabetogenic T cell clones bore receptors encoded by the Vβ5 gene segment (Reich et al., 1989). As these cells are deleted in I-E-expressing mice, this explained the previous findings, although that the

effect was directly caused by endogenous superantigen presentation was not yet understood. Ultimately, all of the studies discussed highlight that the effect of superantigen on the immune repertoire (and the response that an individual can mount) is dependent on the type of superantigen expressed and/or encountered, but also on genetic background, particularly related to the MHC molecules expressed.

In line with this, HLA polymorphisms have been shown to influence superantigen presentation and the outcome of bacterially induced shock (Llewelyn, 2005). Increased binding of superantigen is associated with and increased magnitude of T cell response and the levels of shock induced. As many autoimmune diseases are associated with specific HLA molecules (Zanelli et al., 2000) and particular Vβ use (Acha-Orbea, 1991) it would seem that superantigens could have dramatic effect on the protection from, or susceptibility to, these diseases.

While exposure to exogenous superantigen is normally associated with development of cytokine induced shock (Krakauer, 1999), this is likely to occur when exposed to a single, high dose of antigen. In a setting where chronic exposure to superantigen occurs, the data presented here, and those of others, would argue that rather than developing excessive immune responses, the outcome would be in favour of regulatory immune responses. Evidence points to crucial roles for Treg cells in the resolution of inflammation (McGeachy et al., 2005; Sakaguchi, 2005) and also of immune responses to pathogens (Belkaid and Rouse, 2005). If such a mechanism of superantigen-driven maintenance of Treg frequencies is truly at work in humans, this would represent a subtle further refinement of the “hygiene hypothesis” whereby autoimmune and allergic diseases appear to be minimised, at the cost of increased infectious load (Yazdanbakhsh et al., 2002).

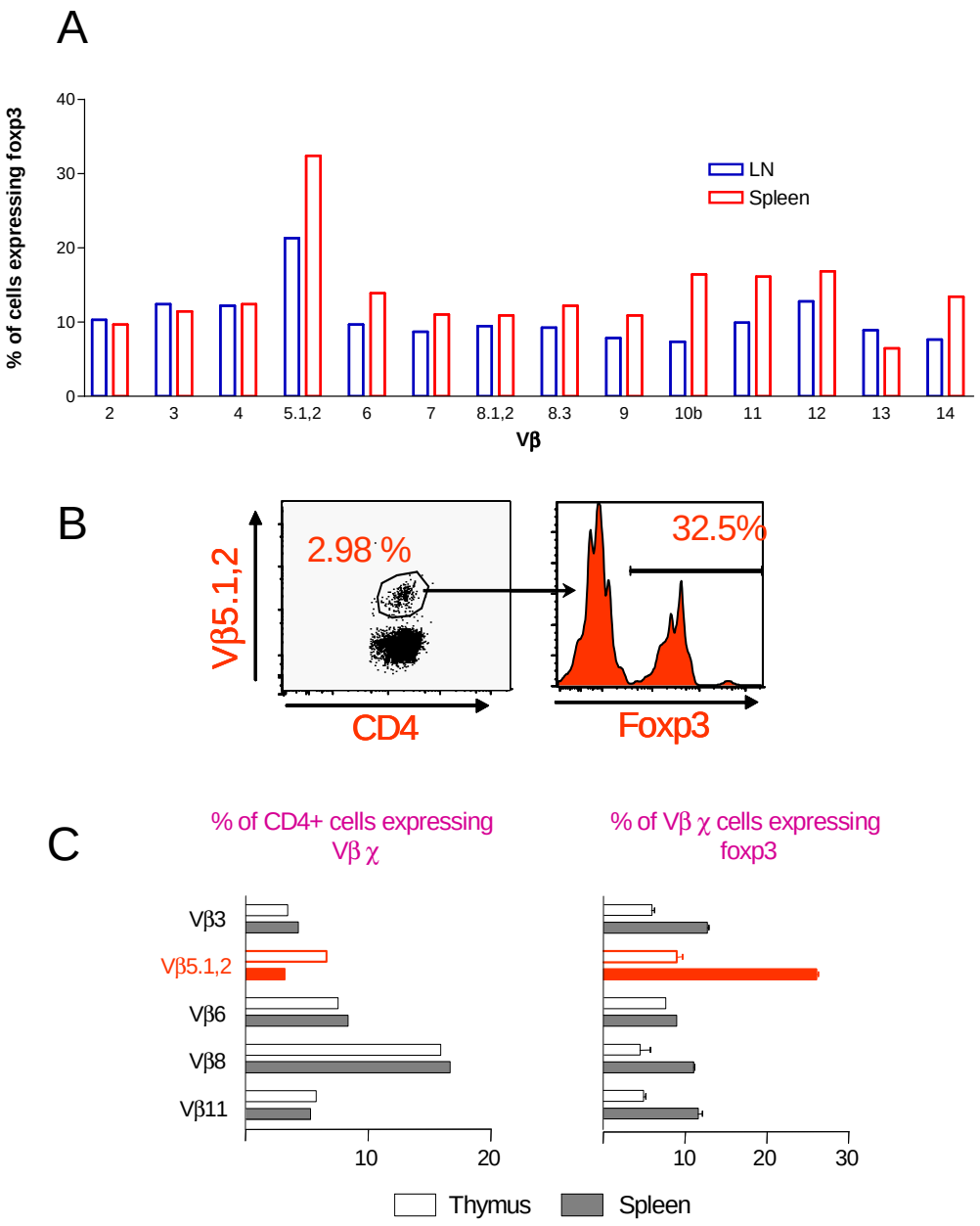
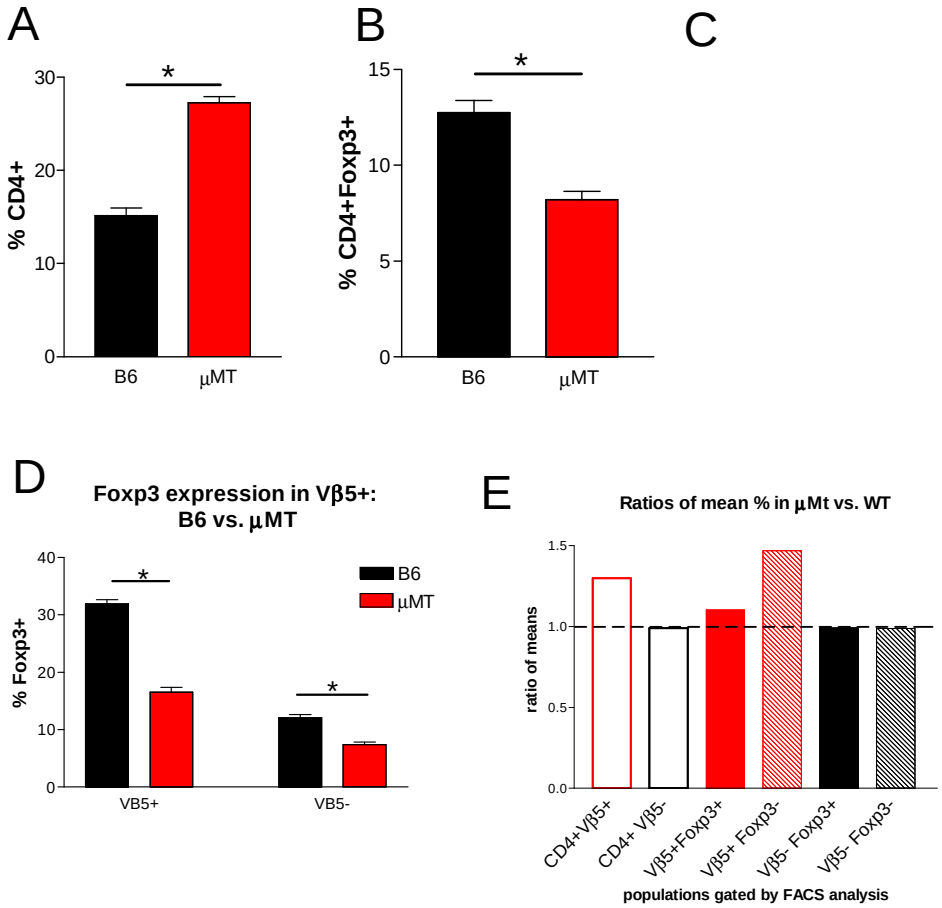


Figure 5.1: CD4+Vβ5+ cells have a high frequency of foxp3+ cells in the periphery of naïve C57BL/6 mice. A: spleen and lymph node samples were stained for expression of CD4, Vβ and Foxp3 expression. B: Representative plot of foxp3 staining on CD4+Vβ5+ splenocytes. C: Thymocytes (gated on CD4+CD8-) were compared with peripheral CD4+CD8- T cells for specific Vβ and foxp3 expression. Errors bars show mean +/- S.D. of three individual mice analysed. Data representative of three separate experiments with similar results.



F

$$\frac{\text{Vβ5+ (\%Foxp3+ in µMT / \%Foxp3+ in WT)}}{\text{Vβ5- (\%Foxp3+ in µMT / \%Foxp3+ in WT)}} = 0.82$$

Figure 5.2: CD4+Vβ5+ T cells from µMT mice have decreased proportions of Foxp3+ cells compared to WT C57BL/6mice. Proportion of A: CD4+, B: CD4+Foxp3+ and C: CD4+Vb5+ cells in WT C57BL/6 mice (black bars) and uMT mice (red bars). D: Proportion of Foxp3+ cells in CD4+Vβ5+ and CD4+Vβ5- compartments. E: Ratio of means in µMT vs. WT cells, calculated from mean percentage of the indicated population in µMT divided by mean % in WT mice. Dotted line represents ratio of 1 (signifying no difference). F: Equation to calculate the ratio of the difference in the proportion of Foxp3+ cells in Vβ5+ vs. Vβ5- populations in µMT vs. WT mice. *=p<0.05. Error bars show mean +/- S.D. of 5 mice/group. Data shows one of two independent experiments.

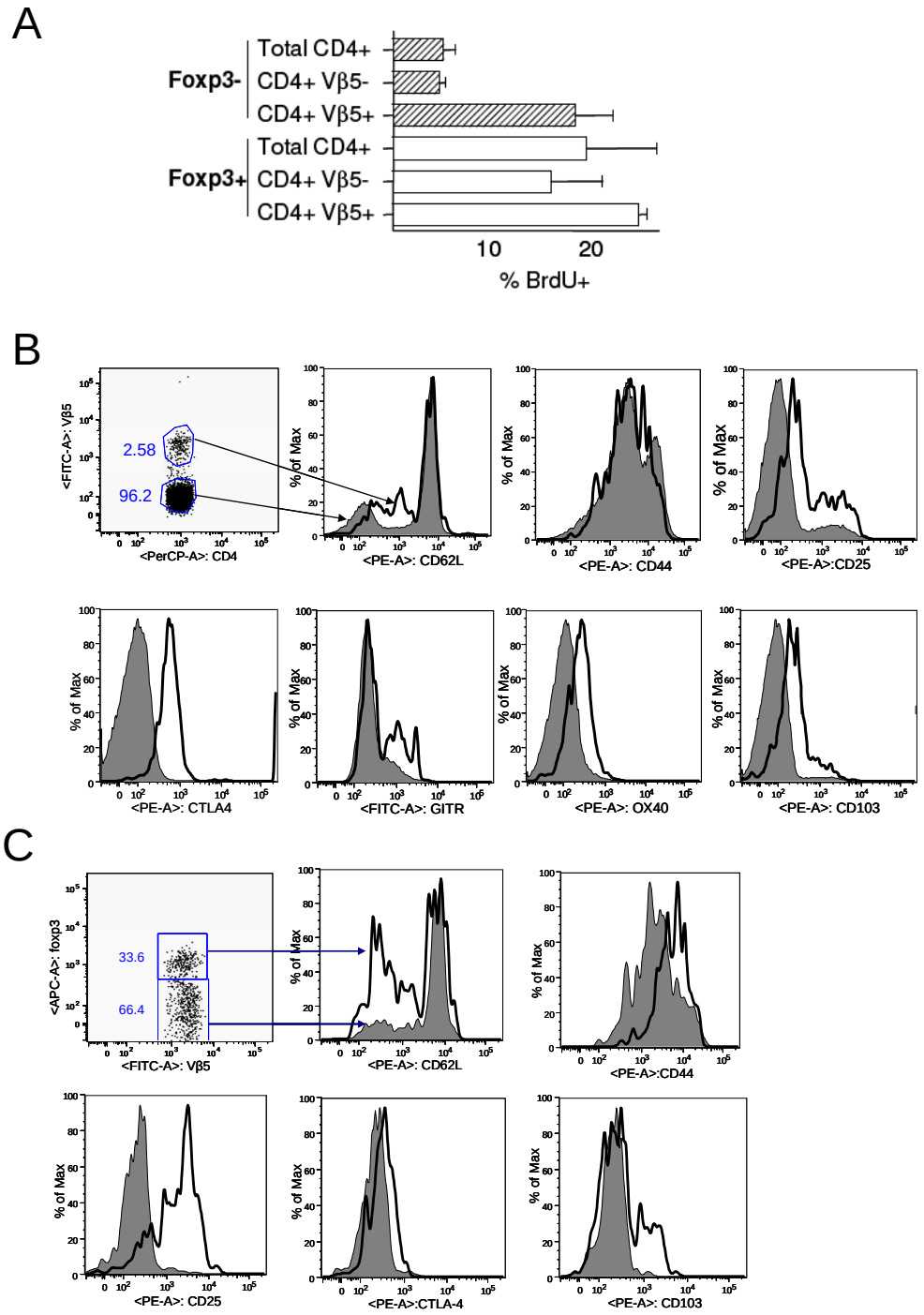


Figure 5.3: Analysis of CD4+Vβ5+ proliferation in vivo and cell surface markers. A: In vivo proliferation of Foxp3- and Foxp3+ populations in vivo, mice were given BrdU i.p. on three consecutive days before sacrifice and staining for FACS analysis. B and C: Cell surface marker expression on Vβ5+ vs Vβ5- (B) and Vβ5+Foxp3+ vs. Vβ5+Foxp3- populations (C). Error bars show mean +/- S.D. of five mice. Plots show one representative of five mice. Data representative of three independent experiments with similar results.

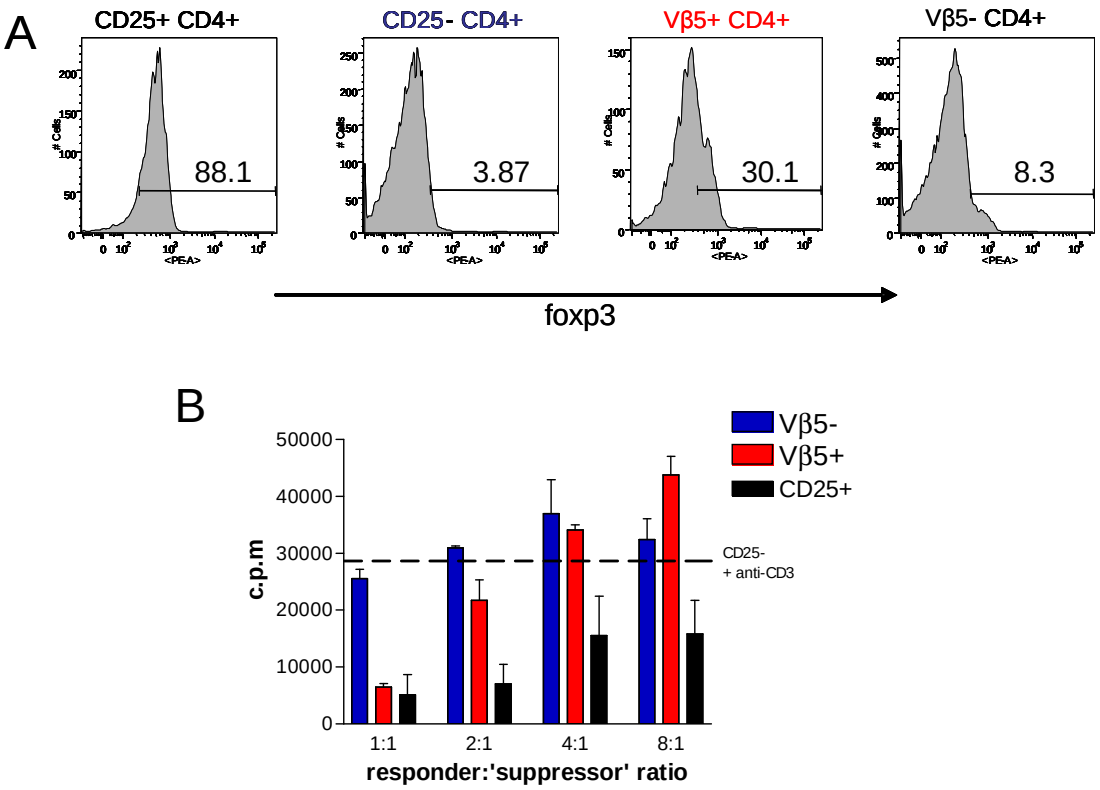


Figure 5.4: In vitro suppression assays to determine suppressive capacity of Vβ5+ cells. A: Indicated populations were sorted from naïve C57BL/6 mice and assessed for Foxp3- expression using FACS (gated based on isotype control for individual samples). B: Suppression of CD4+CD25- cells by sorted CD4+CD25+ cells (black), CD4+Vb5+ cells (red) and CD4+Vb5- cells (blue). Dotted line represents proliferation of CD25- cells to anti-CD3 in the absence of suppressor cells. Error bars show mean +/- S.D. of triplicate wells. Data representative of two independent experiments with similar results.

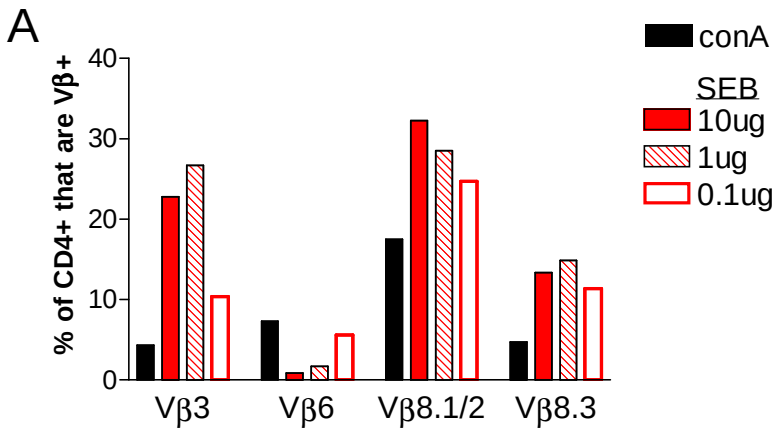


Figure 5.5: SEB selectively induces the expansion of Vβ3+ and Vβ8+ T cells in vitro. Naïve splenocytes from C57BL/6 mice were stimulated with the indicated dose of SEB or conA for 72h in vitro. The proportion of CD4+ cells expressing the indicated Vβ was assessed by FACS. Data representative of triplicate wells, showing data from one of two separate experiments with similar results.

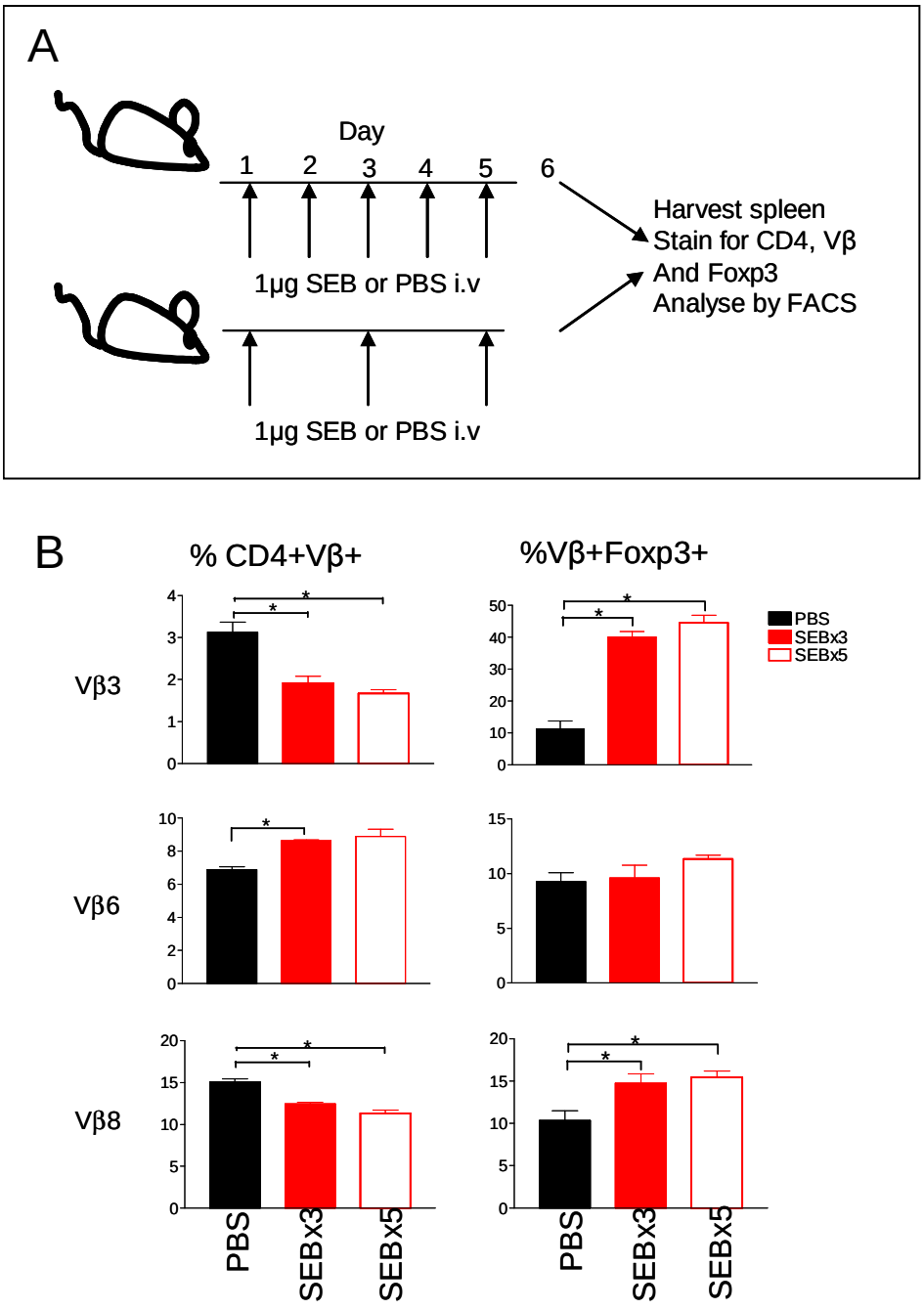


Figure 5.6: SEB induces the depletion of Vβ3+ and Vβ8+ T cells and enhances the frequency of Vβ3 and Vβ8+ Foxp3+ cells in vivo. A: Experimental outline to assess the expansion of TCR Vβ+ cells in response to in vivo administration of SEB. Naïve C57BL/6 mice were given PBS or 1ug SEB i.v. on three or five consecutive days. B: Splenocytes were removed 24h after the last treatment and assessed for levels of TCR Vβ expression (left panels) and Foxp3 expression (right panels). Data representative of three independent experiments, error bars show mean +/- S.D. of 4 individual mice per group. * = $p<0.05$. Data shows one of three repeat experiments with similar results.

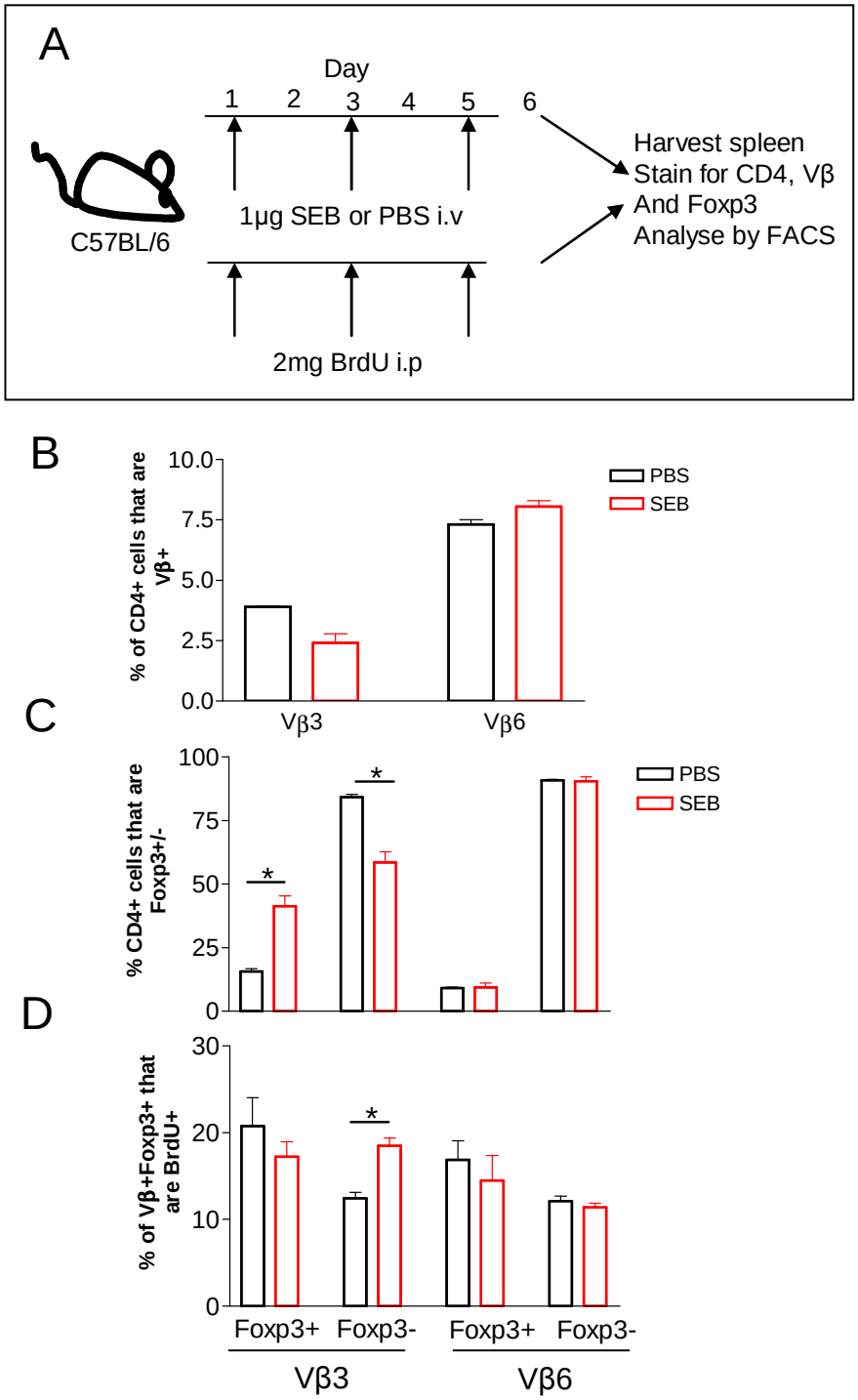


Figure 5.7: SEB stimulation depletes CD4+Vβ3+ T cells and increases the proportion of Vβ3+Foxp3+ cells. A: Experimental outline - naïve C57BL/6 mice were given PBS or 1ug SEB i.v. and BrdU (2mg) i.p. every two days for 5 days. 24h after final treatment splenocytes were removed and CD4+ cells assessed for B: Vβ expression, C: Foxp3 expression and D: BrdU incorporation by FACS. Data representative of three independent experiments. Error bars show mean +/- S.D. of three mice per group, * = $p<0.05$. Data shows one of three repeat experiments with similar results.

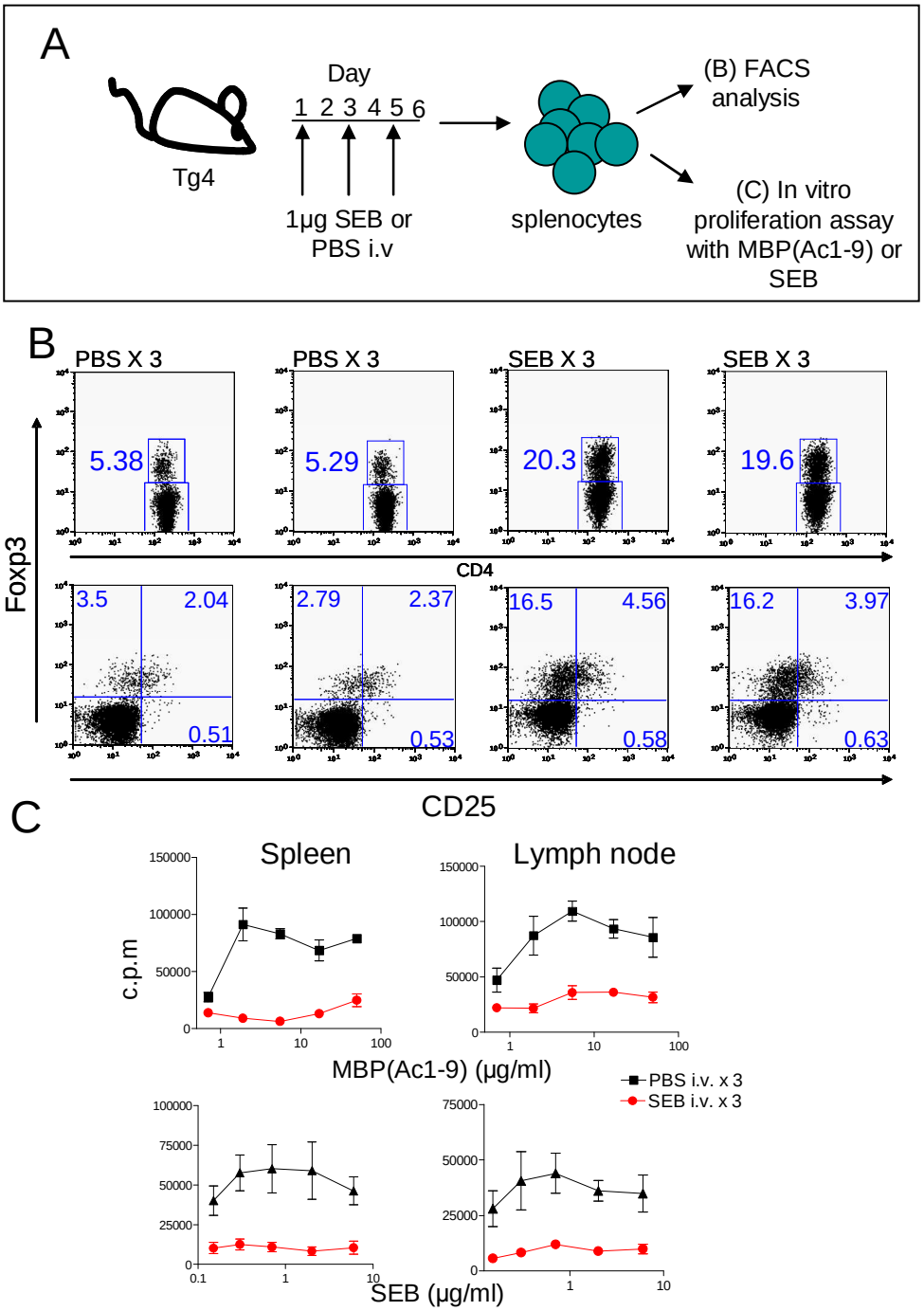


Figure 5.8: Treatment of Tg4 mice with SEB enhances the proportions of Foxp3+ cells and renders the treated Tg4 cells anergic to SEB and MBP stimulation in vitro. Tg4 mice received PBS or 1µg SEB i.v. on three consecutive days and were sacrificed 24h after the last treatment. A: Splenocytes were assessed for Foxp3 and CD25 expression by FACS. Two plots shown per group as a representative of 5 mice per group. B: Splenocytes and lymph nodes were assessed for proliferation in response to MBP(Ac1-9) and SEB in vitro by ^{3}H-thymidine incorporation. Error bars show mean +/- S.D. of 5 mice/group. Data representative of three separate experiments with similar results.

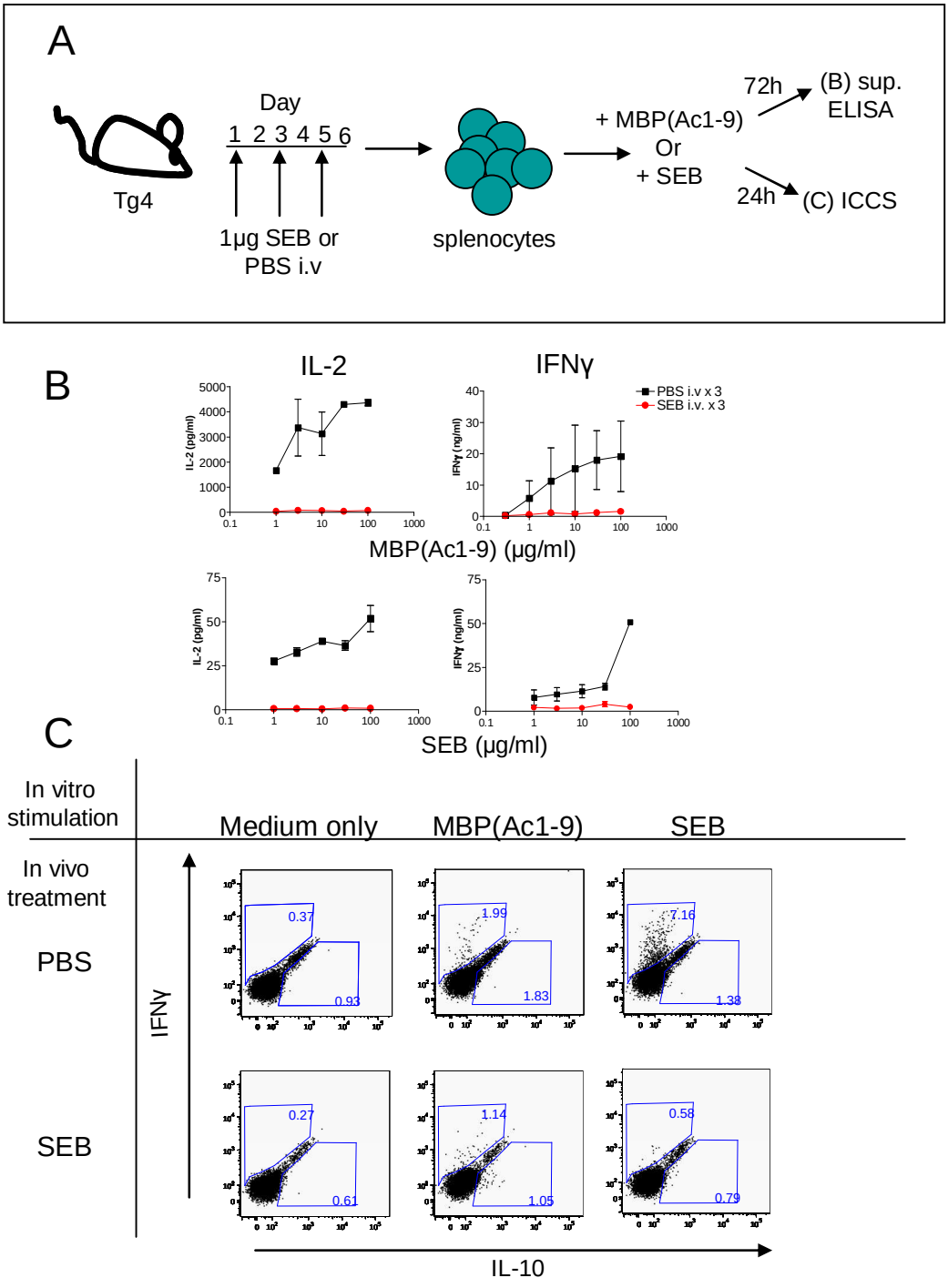


Figure 5.9: Splenocytes from Tg4 mice that have been treated with SEB do not produce cytokines in response to SEB or MBP in vitro. A: Experimental outline. B: splenocytes were stimulated with indicated dose of MBP(Ac1-9) or SEB in vitro for 72h prior to assessment of supernatants by cytokine ELISA or C: splenocytes were stimulated overnight with 50µg MBP(Ac1-9) or SEB for cytokine analysis by ICCS and FACS. Error bars show mean +/- S.D. of individual mice, three mice/group. Plots show one representative of three individual mice analysed. Data representative of three separate experiments with similar results.

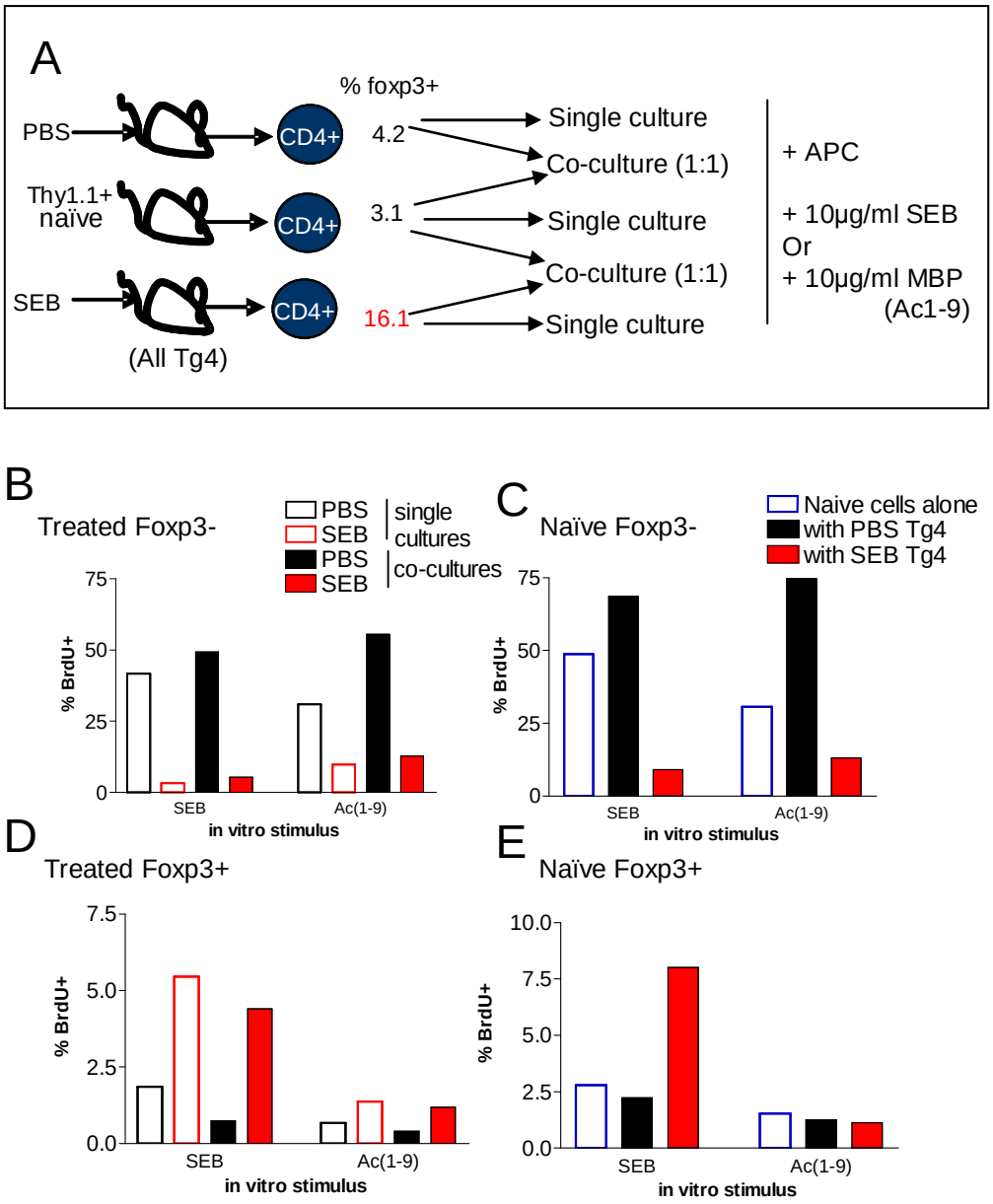


Figure 5.10: CD4+ T cells from SEB treated mice induce proliferation of naive Foxp3+ T cells and suppress proliferation of naïve Foxp3- T cells in response to SEB and MBP(Ac1-9) stimulation in vitro. A: Experimental outline - Tg4 mice were treated with 3x1µg SEB or PBS on three consecutive days prior to the sorting of CD4+ cells from the spleen (% CD4+Foxp3+ cells from each group as assessed by FACS is indicated by number) and pooling samples between groups. B-E: Cells were then cultured alone (open bars) or in co-culture with an equal number of CD4+ cells isolated from naïve Thy1.1+ Tg4 mice (closed bars). Cells were stimulated with MBP(Ac1-9) or SEB (both 10µg/ml) for 48h. BrdU was added to culture for the final 12h to assess proliferation of Foxp3- cells (B and C) and Foxp3+ cells (D and E) by FACS. Figures B and D showing Thy1.1- gated PBS or SEB treated populations, fig. C and E show Thy1.1+ gated naïve Tg4 populations. Data representative of three independent experiments with similar results.

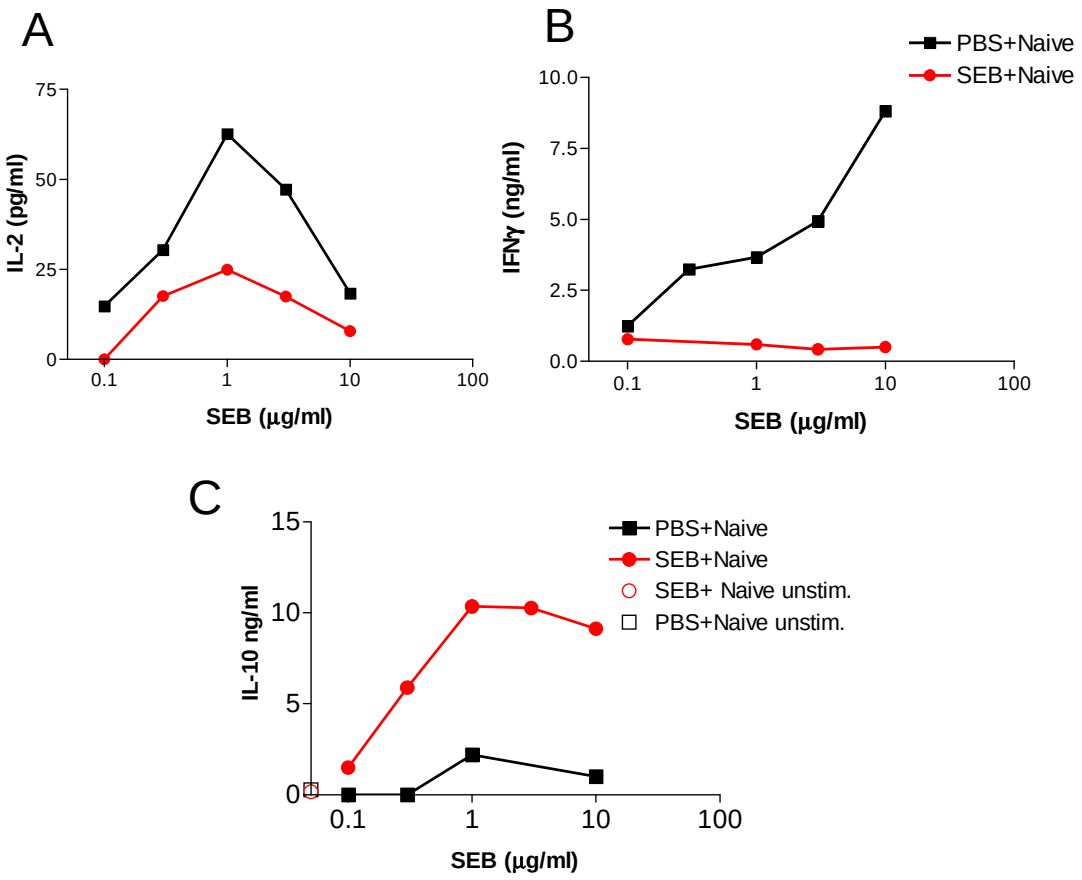


Figure 5.11: Co-culture of naïve Tg4 cells with SEB-experienced Tg4 cells reduced IL-2 and IFNγ production but enhances IL-10 production. Supernatants from co-culture assays shown in Fig. 5.10 were assessed by ELISA for the levels of A: IL-2, B: IFNγ and C: IL-10 in response to SEB stimulation. Data representative of three separate experiments with similar results.

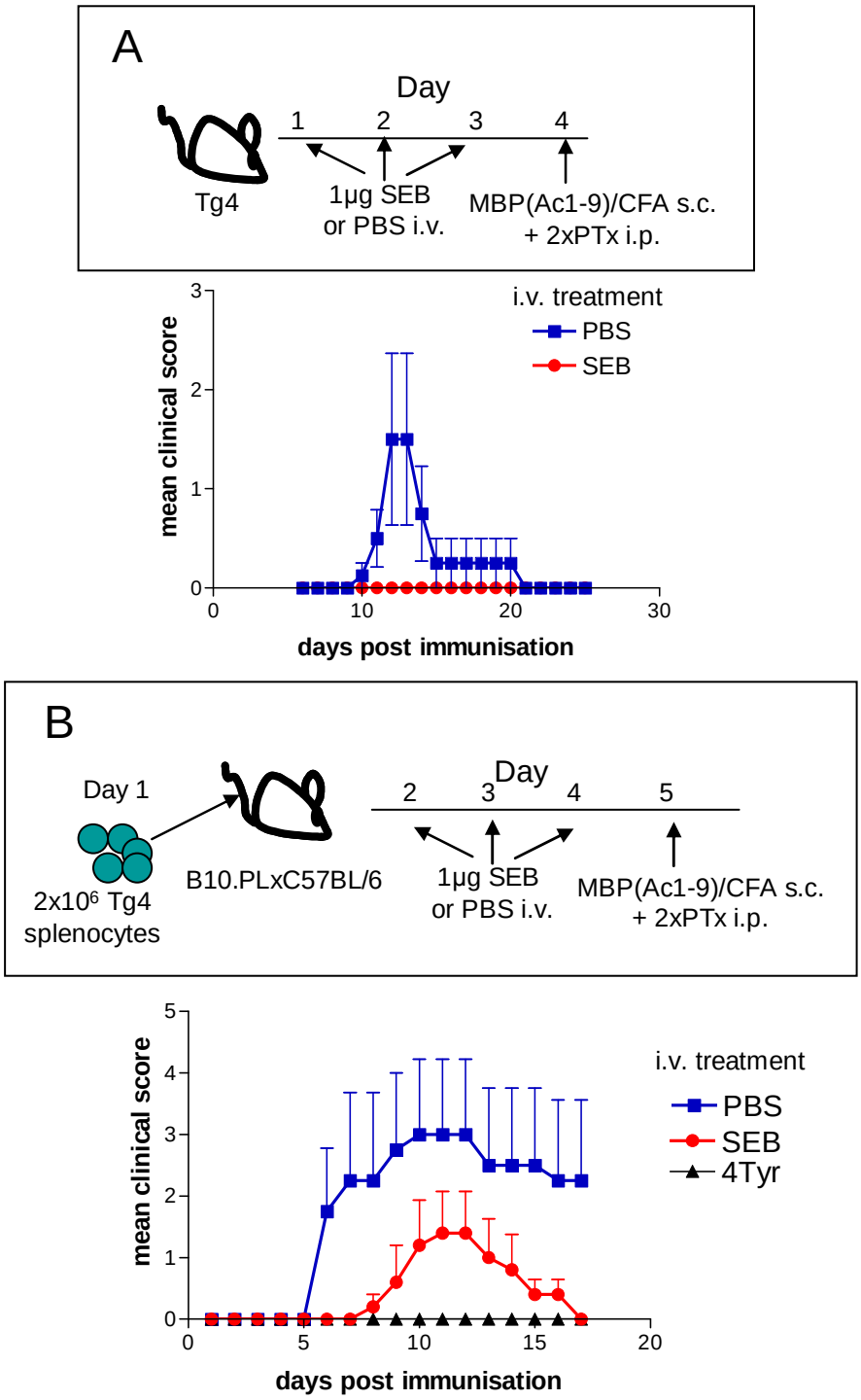


Figure 5.12: Pre-treatment with low dose SEB reduces EAE induced with MBP(Ac1-9) in Tg4 transgenic mice and after adoptive transfer of Tg4 cells. A: Tg4 mice received either PBS or 1µg SEB i.v. on 3 consecutive days prior to the induction of EAE by immunisation with MBP(Ac1-9). B: B10.PLxC57BL/6 mice received a transfer of $2x10^6$ CD4+Tg4 one day before treatment with SEB/PBS and immunisation as in A. Data representative of two independent experiments, error bars show mean +/- S.D of 4 mice per group.

6 General Discussion

Tregs expressing the transcription factor Foxp3 have been shown to be crucial in preventing autoimmunity (Kim et al., 2007; McGeachy et al., 2005). Using a mouse model of MS, EAE, studies from this laboratory have identified that Foxp3+ Tregs accumulate and proliferate in the CNS and are essential for the recovery phase of disease (McGeachy et al., 2005; O'Connor et al., 2007). The data presented in this thesis investigated the antigenic reactivity of Tregs that arise in response to active induction of EAE and the capacity for myelin-reactive Foxp3+ Tregs to be expanded or induced for the prevention and suppression of disease. Furthermore, the effect of superantigenic stimulation on the peripheral Foxp3+ compartment was assessed after identification of specific enhancement in the frequency of Foxp3+ cells in Vβ5+ T cells in the periphery of naïve mice. The data can be summarised as follows;

• Antigen-reactive Tregs can be detected in the periphery and CNS of mice after immunisation with myelin antigens. This occurs both to self and non-self antigens.

• Endogenous expression of self antigen appears to influence the peripheral self antigen-reactive Foxp3+ and Foxp3- repertoires. Furthermore, endogenous antigen expression is not necessary for the expansion/induction of antigen reactive Tregs.

• Pre-treatment with an APL of MBP(Ac1-9) can suppress EAE induced with MBP(Ac1-9) and reduce relapses in EAE induced with PLP(139-151) after adoptive transfer of MBP(Ac1-9)-reactive CD4+ T cells.

• Encounter with SAg enhances the frequency of Foxp3+ T cells in peripheral SAg-reactive T cells and can induce bystander suppression of naïve T cell responses in vitro.

These results have shown that antigen-reactive Tregs can be identified using in vitro based assays. The targeting of disease-relevant antigen-reactive T cells in vivo can suppress disease induced against the initiating antigen, but also induce bystander suppression against distinct antigens. Understanding the antigenic reactivity of Tregs in EAE and MS and the molecular mechanisms behind their selection and expansion in vivo will provide potential therapeutic strategies to prevent or suppress disease.

6.1 Determining the antigenic reactivity of Tregs in EAE and MS

Data from mouse models of autoimmune inflammation have highlighted the enhanced function of antigen-reactive Tregs compared to polyclonal Tregs (Tang et al., 2004b; Tarbell et al., 2004). However, the antigenic-reactivity of Tregs in MS are unknown therefore expansion of disease relevant Tregs requires a greater understanding of the antigenic reactivity of the regulatory cells involved in disease suppression and/or prevention..

Determining the antigens that drive the in vivo activation, proliferation and expansion of Foxp3+ Tregs has proved difficult in the absence of antigen-reactive tetramers. In these studies BrdU-incorporation assays were utilised in combination with Foxp3-staining as a mechanism to detect the proliferation of Foxp3+ cells in vitro. However, this assay was limited, as the proliferation of Foxp3+ cells was almost universally accompanied by Foxp3- cell proliferation. It is therefore difficult to rule out that Treg proliferation was induced by the antigen in vitro, and not in response to a Foxp3- derived factor, such as IL-2. Further refinement of this protocol would be necessary to definitively conclude that the Tregs in culture were indeed antigen-reactive. However, the data presented here is in agreement with those of the Kuchroo group (Korn et al., 2007) using antigen-specific tetramers.

Determining the antigenic-reactivity of Tregs in humans will be much more complex than in the models used in this study. The initiating antigens for disease are unknown and as many patients have undergone substantial de-myelination before clinical manifestations, a number of myelin antigens are likely to be involved. However, the identification of auto-reactive T cells in MS patients has been shown in the blood of MS patients (Hellings et al., 2001; Zhang et al., 1994); this could also be used to determine the antigenic reactivity of Foxp3+ cells from the peripheral blood of patients. One major caveat of human studies is the accessibility to Tregs from the site of inflammation. While most of the studies on human Tregs have isolated these cells from peripheral blood, it is likely that Tregs from the CNS have refined specificities and may differ from peripheral Tregs.

6.2 Therapeutic targeting of Tregs in MS

Transfer of large number of naïve polyclonal Tregs has been shown to reduce the severity of EAE (Kohm et al., 2002; Zhang et al., 2004). Previous data from our laboratory has shown that depletion of Tregs using anti-CD25 antibody resulted in exacerbated EAE, while depletion after the initial phase of disease prolonged the disease course (McGeachy et al., 2005; Stephens et al., 2005). These studies highlighted the key function of Tregs in EAE, therefore their role in MS has also been investigated.

CD4+CD25^{hi} cells from human peripheral blood represent a regulatory T cell population with properties similar to those isolated from mice (Baecher-Allan et al., 2001; Dieckmann et al., 2001). CD4+CD25+ cells from MS patients were shown to be present at similar numbers relative to healthy controls however, Tregs from MS patients were shown to have reduced functional suppressive capacities (Haas et al., 2005; Viglietta et al., 2004). Immunotherapy that enhances the capacity of Tregs to suppress auto-aggressive responses would therefore be beneficial.

Our data demonstrated that MBP(Ac1-9) reactive Tregs can be expanded in vitro and suppress MBP(Ac1-9)-induced EAE when transferred at low cell numbers. The expansion of human myelin reactive Tregs in vitro has also now been shown (Arbour et al., 2006). The in vitro expansion and transfer of myelin reactive Tregs as therapy would be a technically difficult and expensive method to treat MS patients. However, this has provided a model to test the capacity of myelin-reactive T cells in vivo. We used this model to determine that MBP(Ac1-9) reactive Tregs could reduce relapses of disease induced against the 139-151 epitope of PLP, a distinct myelin antigen.

To overcome the demands of cellular therapies, targeting Treg expansion directly in vivo is a possibility for MS treatment. Certain protocols of antigen administration have been shown to favour the induction and expansion of Foxp3+ T cells, such as low antigen dose that provides continual antigenic-stimulation (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). We utilised an APL of MBP(Ac1-9), 4Tyr, that had previously been shown to have profound tolerogenic properties in EAE (Burkhart et al., 1999; Sundstedt et al., 2003). Treatment of mice that had received Tg4 cells with this peptide suppressed disease induced against MBP(Ac1-9) and PLP139-151. However, no Foxp3+ cells were detected in these studies. Nonetheless, the fact that Tregs reactive against one antigen can induce suppression against a distinct antigen has important implications in MS. Similar to mouse models, human studies have suggested that epitope spreading can also occur in MS (Goebels et al., 2000). Although not every antigen can be identified in MS, it is likely that identification of key epitopes that the immune system is recognising will allow the targeting of these cells towards a regulatory phenotype, or the expansion of nTregs recognising these antigens. Bystander suppression mechanisms will then help generate a regulatory and suppressive environment in the CNS.

Some studies have suggested that Tregs do not function optimally in settings of inflammation (Pasare and Medzhitov, 2003). One study has suggested that although Tregs accumulate in the CNS, they fail to suppress auto-aggressive T cell responses (Korn et al., 2007). Data from our group have also suggested that Tregs do not suppress

Th17 responses, but can suppress Th1 responses (O'Connor et al., 2007). Combination protocols promoting Tregs and reducing inflammation will therefore provide optimal therapies for CNS inflammation, although specific targeting of the CNS environment will be difficult and may still involve some aspect of general immunosuppression. Rapamycin, which inhibits the response to IL-2, has been shown to preferentially promote Treg expansion while inhibiting effector T cell proliferation (Battaglia et al., 2006c). Combination peptide and rapamycin therapy would be a potential therapy that requires investigation.

Peptide therapy as a treatment for human inflammatory conditions has shown some promise (Larche and Wraith, 2005). However, the molecular mechanisms that result in tolerance require deeper understanding to ensure that only Tregs are targeted and that activation or enhancement of Teff cells is not induced. Furthermore, the longevity of the tolerance must be addressed. Potentially, patients may require continual doses of peptide to maintain suppression and developing protocols to induce long-term tolerance to many CNS-derived antigens will be required to circumvent this problem.

6.3 Superantigens and their implications in autoimmunity

Studies on the TCR use of Foxp3+ Tregs lead us to investigate the finding that Vβ5+ T cells were under-represented in total CD4+ populations and ~30% of these cells were Foxp3+. Mmtv-derived superantigen-mediated deletion of T cells bearing particular TCRβ chains had been extensively documented in the 1990's (Lobo-Yeo and Lamb, 1993; Scherer et al., 1995) and accounted for the loss of Vβ5+ cells from the total CD4+ compartment. However, while some studies had reported that the remaining Vβ5+ cells had regulatory properties, Foxp3-expression had not been documented.

The data presented here demonstrates that exposure to superantigen (both endogenous and exogenous) results in the proliferation of T cells bearing particular Vβ chains,

followed by their elimination. However, Foxp3+ cells were refractory to primary superantigen stimulation and were presumable spared from deletion. Overall, superantigen encounter skews the SAg-reactive population towards a regulatory phenotype by enhancing the frequency of Foxp3+ cells. Furthermore, SAg-exposed T cells induce suppression of primary T cell responses, potentially via the release of IL-10 and favour the balance of the immune system towards regulation.

As superantigens target T cells based on the expression of TCR β chains, they can affect a vast proportion of all T cells; much larger than the population of T cells that would be affected by antigenic encounter. In this way, superantigens can have profound consequences on the generation of subsequent immune responses, including autoimmune responses.

Exposure to SEB can have differential effects in EAE, depending on the strain of mouse used, initiating antigen and time of administration (Brocke et al., 1993; Kuschnaroff et al., 1999). MBP-reactive T cell clones from the blood of MS patients have been shown to be reactive to superantigens (Zhang et al., 1995). Furthermore, the association of MS with viral infection, such as EBV, have suggested that exposure to superantigens may be a risk factor for MS (Tai et al., 2008). However, the data shown here suggests that chronic exposure to superantigen may result in enhanced immune regulation and suppress autoimmunity. It is likely the timing and intensity of exposure (high dose vs. chronic low dose stimulation) to superantigens will affect the outcome that these antigens have in autoimmunity.

While superantigens do not provide potential therapeutic strategies for the prevention of autoimmunity, they can be used to examine the molecular responses of T cells in vivo. Clearly, the data presented here shows that Teff and Tregs respond differently to the same (super)antigen. Understanding the mechanisms behind the selective elimination of Teff compared to the lack of proliferation and hence survival of Tregs is of great

interest, particularly in diseases such as MS, where the selective survival of Tregs and elimination of effector T cells would be advantageous.

This project provides insight into the antigenic reactivity and functional capacity of CNS-relevant Foxp3+ Tregs in models of EAE. The molecular requirements for Treg selection and expansion and their response to superantigen stimulation highlight important properties of Foxp3+ Tregs that must be considered to translate Treg-based therapies from mouse models to human diseases.

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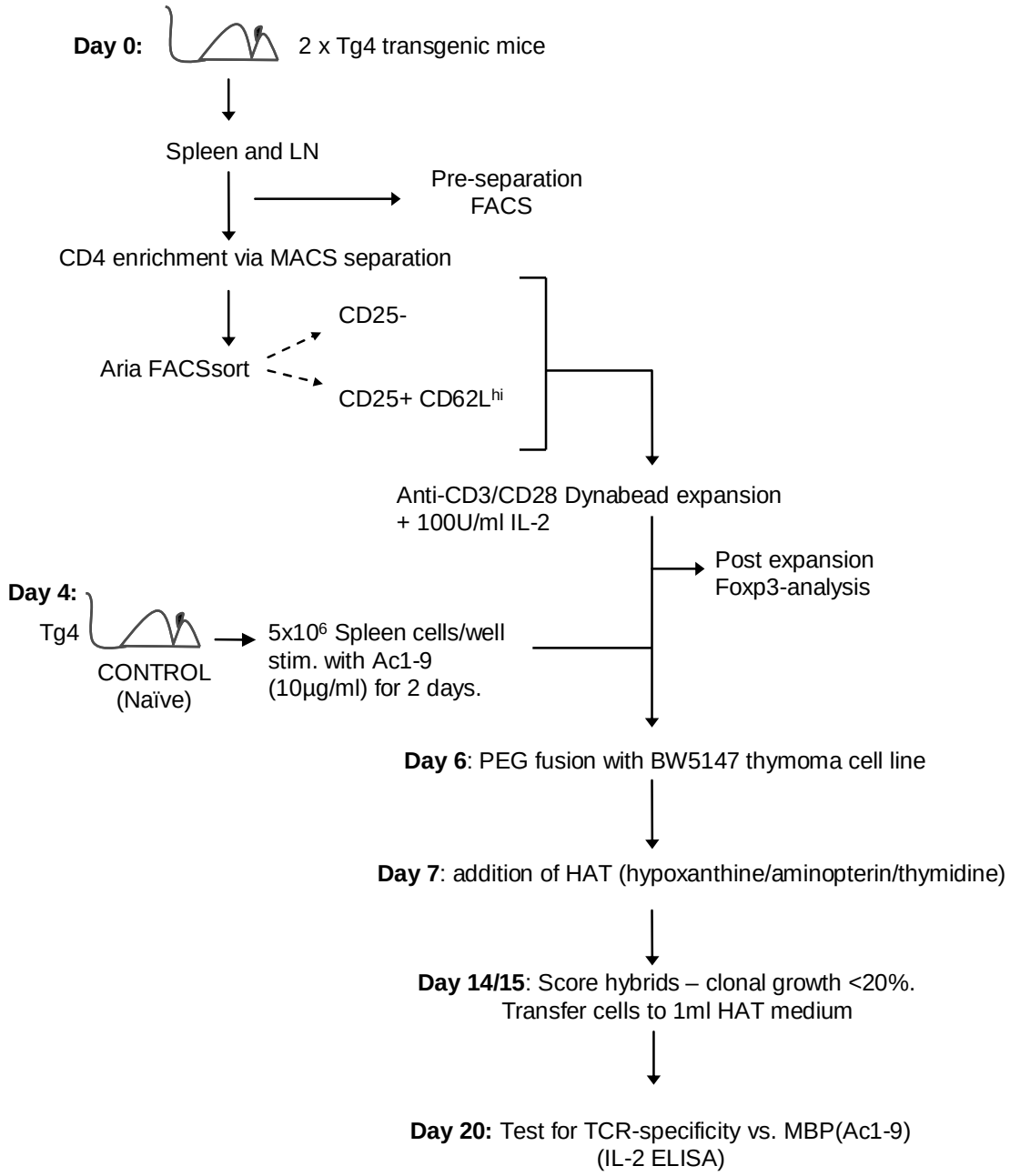
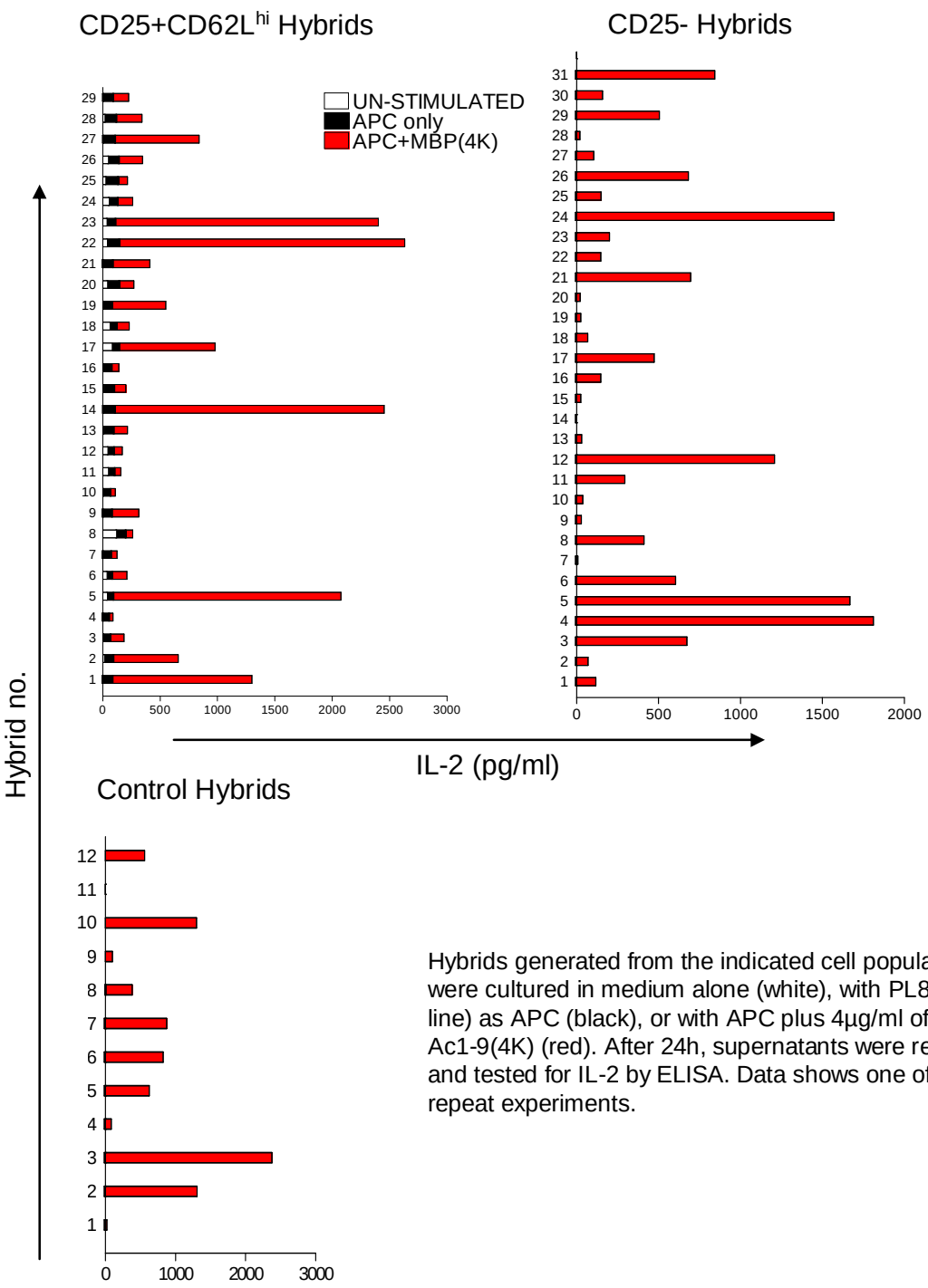
8 Appendices

Appendix 1A: Outline of the protocol to generate Treg hybridomas

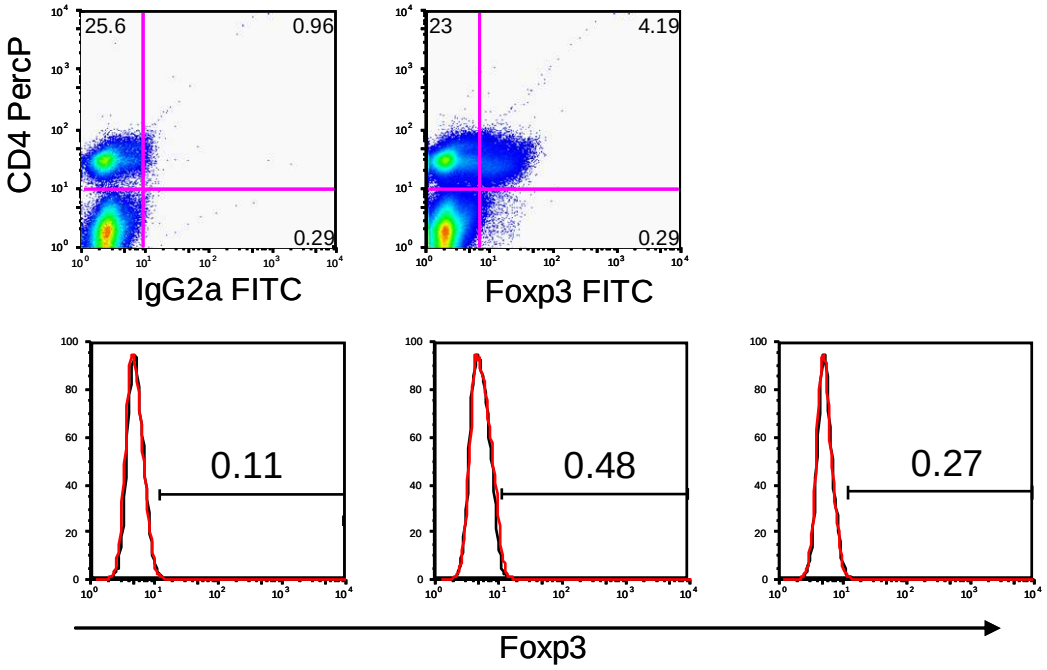


Appendix 1B: IL-2 ELISA showing Tg4 hybrid responsiveness to MBP(Ac1-9)



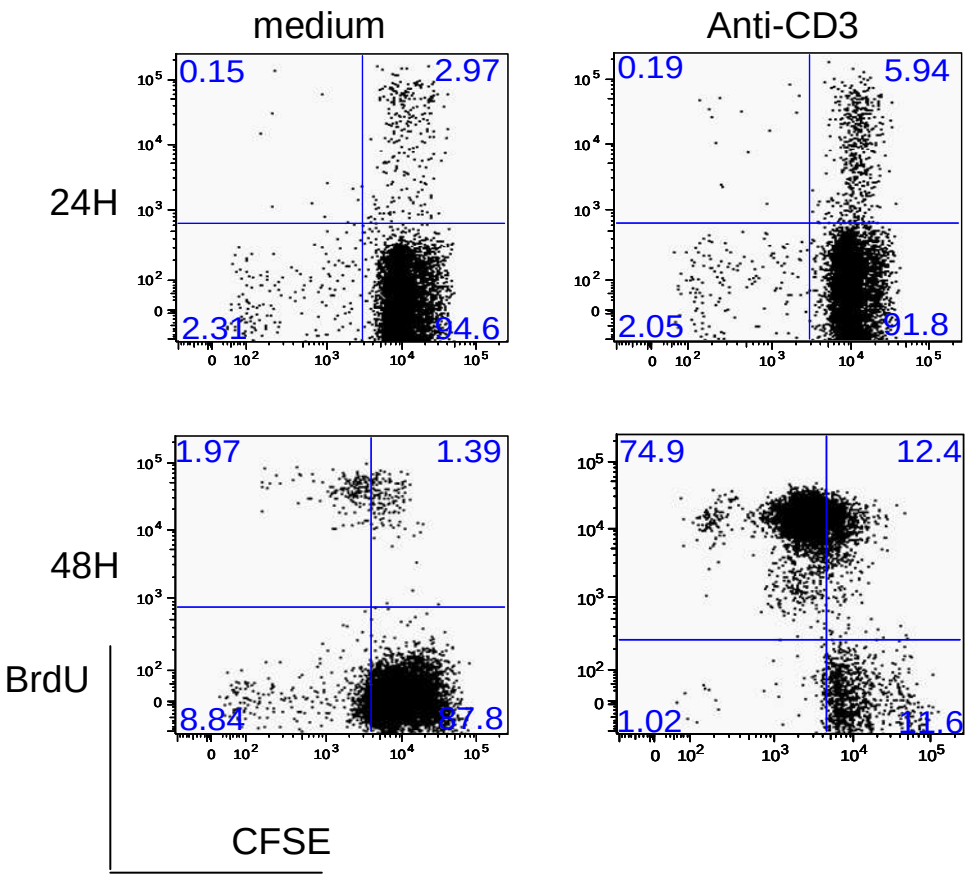
Hybrids generated from the indicated cell populations were cultured in medium alone (white), with PL8 (B cell line) as APC (black), or with APC plus 4µg/ml of MBP Ac1-9(4K) (red). After 24h, supernatants were removed and tested for IL-2 by ELISA. Data shows one of three repeat experiments.

Appendix 1C: Foxp3-expression by Tg4 Treg hybrids



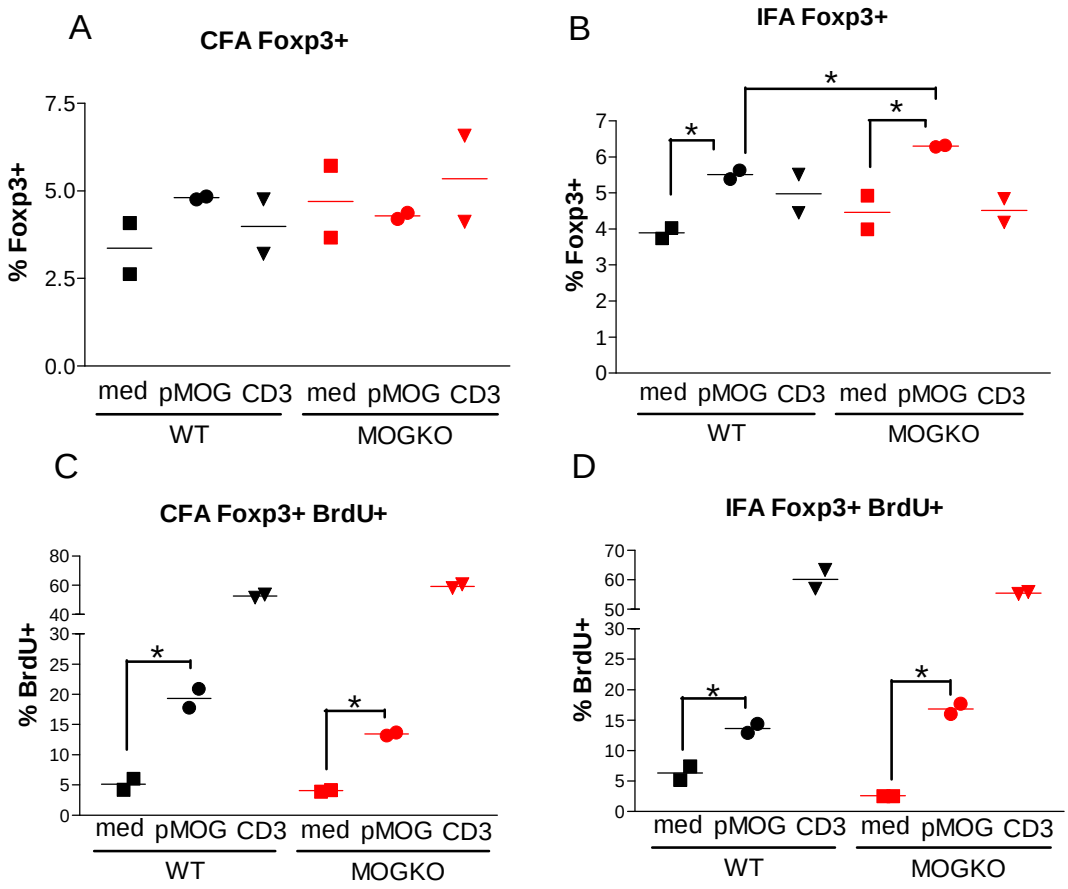
Top panels: control Tg4 cells (un-sorted, whole spleen Tg4) - isotype control (left) and foxp3 expression (right). Bottom panels: three representative plots of Foxp3 expression in fused CD25+CD62L^{hi} Tg4 hybrids, black line = isotype control.

Appendix 2: Double staining for BrdU and CFSE to show BrdU incorporation is a suitable measurement of cell division.



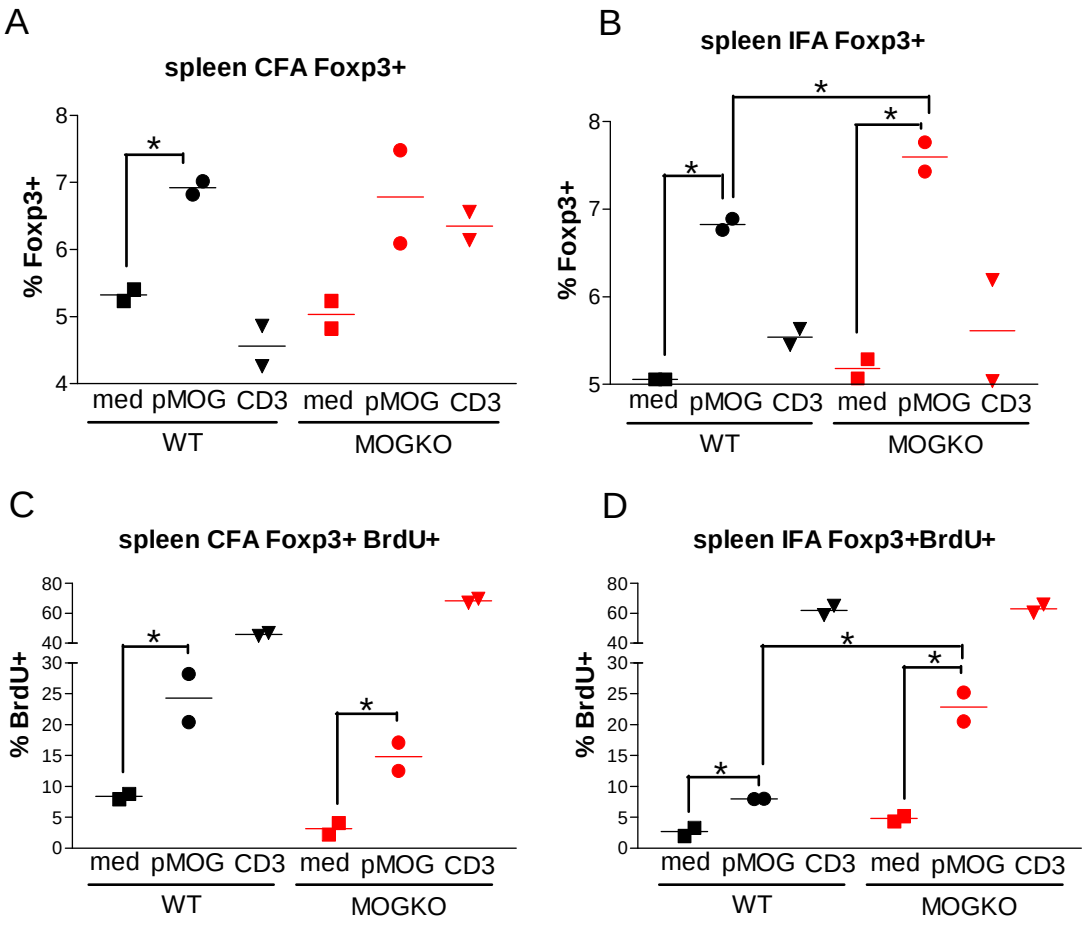
Splenocytes from naïve C57BL/6 mice were labelled with CFSE and stimulated for 24 (top panels) and 48h (bottom panels) with anti-CD3 (1µg/ml final concentration). For the final 12h of each culture BrdU was added to assess BrdU incorporation and CFSE dilution simultaneously via FACS. Plots show a representative of 3 separate wells showing similar results.

Appendix 3A: Assessing the effect of CFA vs. IFA immunisation on determining antigen-reactivity ex vivo (Percentages).



WT and MOGKO mice were immunised with pMOG in CFA or IFA. 10 days later, DLN were removed and assessed for in vitro reactivity to pMOG. Top panels: Proportion of Foxp3+ cells in cultures from CFA-immunised (A) and IFA immunised (B) mice. Bottom panels: Proportion of Foxp3+BrdU+ cells in culture from CFA immunised (C) and IFA immunised (D) mice. *p<0.05. Data show duplicate wells of pooled samples between groups and represents one of two repeat experiments with similar results.

Appendix 3B: Assessing the effect of CFA vs. IFA immunisation on determining antigen-reactivity ex vivo (cell numbers).



WT and MOGKO mice were immunised with pMOG in CFA or IFA. 10 days later, DLN were removed and assessed for in vitro reactivity to pMOG. Top panels: Proportion of Foxp3+ cells in cultures from CFA-immunised (A) and IFA immunised (B) mice. Bottom panels: Proportion of Foxp3+BrdU+ cells in culture from CFA immunised (C) and IFA immunised (D) mice. *$p<0.05$. Data show duplicate wells of pooled samples between groups and represents one of two repeat experiments with similar results.